05-31-00

Practitioner's Docket No. 701039-48802 C *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of
Inventor(s): Michael KLAGSBRUN, Shay SOKER, Hua-Quan MIAO, Seiji TAKASHIMA.

WARNING: *37 C.F.R. § 1.41(a)(1) points out:*

"(a) A patent is applied for in the name or names of the actual inventor or inventors.

(1) The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.63, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(i) is filed supplying or changing the name or names of the inventor or inventors."

For (title): ANTAGONISTS OF NEUROPILIN RECEPTOR FUNCTION AND USE THEREOF

CERTIFICATION UNDER 37 C.F.R. 1.10*
*(Express Mail label number is **mandatory**.)*
(Express Mail certification is optional.)

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on this date 30 May 2000, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number EK571074376US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Nicholas A. Zachariades
(type or print name of person mailing paper)

N Zachariades

Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

****WARNING:*** *Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. 1.10(b).*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.*

1. Type of Application

This new application is for a(n)

(check one applicable item below)

[X] Original (nonprovisional)
[ ] Design
[ ] Plant

WARNING: ***Do not*** *use this transmittal for a completion in the U.S. of an International Application under 35 U.S.C. 371(c)(4), unless the International Application is being filed as a divisional, continuation or continuation-in-part application.*

WARNING: ***Do not*** *use this transmittal for the filing of a provisional application.*

NOTE: If one of the following 3 items apply, then complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF A PRIOR U.S. APPLICATION CLAIMED and a NOTIFICATION IN PARENT APPLICATION OF THE FILING OF THIS CONTINUATION APPLICATION.

[ ] Divisional.
[X] Continuation.
[ ] Continuation-in-part (C-I-P).

2. Benefit of Prior U.S. Application(s) (35 U.S.C. 119(e), 120, or 121)

NOTE: A nonprovisional application may claim an invention disclosed in one or more prior filed copending nonprovisional applications or copending international applications designating the United States of America. In order for a nonprovisional application to claim the benefit of a prior filed copending nonprovisional application or copending international application designating the United States of America, each prior application must name as an inventor at least one inventor named in the later filed nonprovisional application and disclose the named inventor's invention claimed in at least one claim of the later filed nonprovisional application in the manner provided by the first paragraph of 35 U.S.C. 112. Each prior application must also be:

(i) An international application entitled to a filing date in accordance with PCT Article 11 and designating the United States of America; or

(ii) Complete as set forth in § 1.51(b); or

(iii) Entitled to a filing date as set forth in § 1.53(b) or § 1.53(d) and include the basic filing fee set forth in § 1.16; or

(iv) Entitled to a filing date as set forth in § 1.53(b) and have paid therein the processing and retention fee set forth in § 1.21(l) within the time period set forth in § 1.53(f).

37 C.F.R. § 1.78(a)(1).

NOTE If the new application being transmitted is a divisional, continuation or a continuation-in-part of a parent case, or where the parent case is an International Application which designated the U.S., or benefit of a prior provisional application is claimed, then check the following item and complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

WARNING: *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. 120, 121*

or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. 120, 121 or 365(c). (35 U.S.C. 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.

WARNING: *When the last day of pendency of a provisional application falls on a Saturday, Sunday, or Federal holiday within the District of Columbia, any nonprovisional application claiming benefit of the provisional application* ***must*** *be filed prior to the Saturday, Sunday, or Federal holiday within the District of Columbia. See 37 C.F.R. § 1.78(a)(3).*

[X] The new application being transmitted claims the benefit of prior U.S. application(s). Enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

3. Papers Enclosed

A. Required for Filing Date under 37 C.F.R. § 1.53(b) (Regular) or 37 C.F.R. § 1.153 (Design) Application

46 Pages of Specification
2 Pages of Claims
21 Sheets of Drawing

WARNING: ***DO NOT*** *submit original drawings. A high quality copy of the drawings should be supplied when filing a patent application. The drawings that are submitted to the Office must be on strong, white, smooth, and non-shiny paper and meet the standards according to § 1.84. If corrections to the drawings are necessary, they should be made to the original drawing and a high-quality copy of the corrected original drawing then submitted to the Office. Only one copy is required or desired. For comments on proposed then-new 37 C.F.R. 1.84, see Notice of March 9, 1988 . (1990 O.G. 57-62).*

NOTE: "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page. . ." 37 C.F.R. § 1.84(c)).

(complete the following, if applicable)

[ ] The enclosed drawing(s) are photograph(s), and there is also attached a "PETITION TO ACCEPT PHOTOGRAPH(S) AS DRAWING(S)." 37 C.F.R. § 1.84(b).

[ ] Formal
[X] Informal

B. Other Papers Enclosed

____ Pages of declaration and power of attorney
1 Pages of Abstract
9 Other (Sequence Listing)

4. Additional Papers Enclosed

[ ] Amendment to claims

[ ] Cancel in this applications claims ________________ before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

[ ] Add the claims shown on the attached amendment. (Claims added have been numbered consecutively following the highest numbered original claims.)

[ ] Preliminary Amendment
[ ] Information Disclosure Statement (37 C.F.R. § 1.98)
[ ] Form PTO-1449 (PTO/SB/08A and 08B)
[ ] Citations
[ ] Declaration of Biological Deposit
[ ] Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence.
[ ] Authorization of Attorney(s) to Accept and Follow Instructions from Representative
[ ] Special Comments
[ ] Other

5. Declaration or Oath (including power of attorney)

NOTE: A newly executed declaration is not required in a continuation or divisional application provided the prior nonprovisional application contained a declaration as required, the application being filed is by all or fewer than all the inventors named in the prior application, there is no new matter in the application being filed, and a copy of the executed declaration filed in the prior application (showing the signature or an indication thereon that it was signed) is submitted. The copy must be accompanied by a statement requesting deletion of the names of person(s) who are not inventors of the application being filed. If the declaration in the prior application was filed under § 1.47 then a copy of that declaration must be filed accompanied by a copy of the decision granting § 1.47 status or, if a nonsigning person under § 1.47 has subsequently joined in a prior application, then a copy of the subsequently executed declaration must be filed. See 37 C.F.R. § 1.63(d)(1)-(3).

NOTE: A declaration filed to complete an application must be executed, identify the specification to which it is directed, identify each inventor by full name, including the family name, and at least one given name without abbreviation together with any other given name or initial, and the residence, post office address and country of citizenship of each inventor, and state whether the inventor is a sole or joint inventor. 37 C.F.R. § 1.63(a)(1)-(4).

[ ] Enclosed

Executed by

(check **all** *applicable boxes)*

[ ] inventor(s).
[ ] legal representative of inventor(s). 37 C.F.R. § 1.42 or 1.43.
[ ] joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.

[ ] This is the petition required by 37 C.F.R. § 1.47 and the statement required by 37 C.F.R. § 1.47 is also attached. See item 13 below for fee.

[X] Not Enclosed.

NOTE: *Where the filing is a completion in the U.S. of an International Application, or where the completion of the U.S. application contains subject matter in addition to the International Application, the application may be treated as a continuation or continuation-in-part, as the case may be, utilizing ADDED PAGE FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION CLAIMED.*

[ ] Application is made by a person authorized under 37 C.F.R. 1.41(c) on behalf of *all* the above named inventor(s).

(The declaration or oath, along with the surcharge required by 37 C.F.R. § 1.16(e), can be filed subsequently).

[ ] Showing that the filing is authorized.
(not required unless called into question. 37 C.F.R. § 1.41(d))

6. Inventorship Statement

WARNING: *If the named inventors are each not the inventors of all the claims an explanation, including the ownership of the various claims at the time the last claimed invention was made, should be submitted.*

The inventorship for all the claims in this application are:

[ ] The same.

or

[ ] Not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made,
[ ] is submitted.
[ ] will be submitted.

7. Language

NOTE: *An application including a signed oath or declaration may be filed in a language other than English. An English translation of the non-English language application and the processing fee of $130.00 required by 37 C.F.R. § 1.17(k) is required to be filed with the application, or within such time as may be set by the Office. 37 C.F.R. § 1.52(d).*

[X] English
[ ] Non-English

[ ] The attached translation includes a statement that the translation is accurate. 37 C.F.R. § 1.52(d).

8. Assignment

[X] An assignment of the invention to Children's Medical Center Corporation

[ ] is attached. A separate [ ] "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or [ ] FORM PTO 1595 is also attached.

[X] will follow.

NOTE: "If an assignment is submitted with a new application, send two separate letters-one for the application and one for the assignment" Notice of May 4, 1990 (1114 O.G. 77-78).

WARNING: *A newly executed "STATEMENT UNDER 37 C.F.R. § 3.73(b)" must be filed when a continuation-in-part application is filed by an assignee. Notice of April 30, 1993, 1150 O.G. 62-64.*

9. Certified Copy

Certified copy(ies) of application(s)

Country	Appln. no.	Filed
Country	Appln. no.	Filed
Country	Appln. no.	Filed

from which priority is claimed

[ ] is (are) attached.

[ ] will follow.

NOTE: The foreign application forming the basis for the claim for priority must be referred to in the oath or declaration. 37 C.F.R. § 1.55(a) and 1.63.

NOTE: This item is for any foreign priority for which the application being filed directly relates. If any parent U.S. application or International Application from which this application claims benefit under 35 U.S.C. 120 is itself entitled to priority from a prior foreign application, then complete item 18 on the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

10. Fee Calculation (37 C.F.R. § 1.16)

A. [X] Regular application

CLAIMS AS FILED					
Claims	Number Filed	Basic Fee Allowance	Number Extra	Rate	Basic Fee 37 C.F.R. § 1.16(a) $760.00
Total Claims (37 C.F.R. § 1.16(c))		- 20 =	x	$ 18.00	
Independent Claims (37 C.F.R. § 1.16(b))		- 3 =	x	$ 78.00	
Multiple Dependent Claim(s), if any (37 C.F.R. § 1.16(d))			+	$260.00	

[ ] Amendment cancelling extra claims is enclosed.
[ ] Amendment deleting multiple-dependencies is enclosed.
[ ] Fee for extra claims is not being paid at this time.

NOTE: If the fees for extra claims are not paid on filing they must be paid or the claims cancelled by amendment, prior to the expiration of the time period set for response by the Patent and Trademark Office in any notice of fee deficiency. 37 C.F.R. § 1.16(d).

Filing Fee Calculation $___________

B. [ ] Design application
($310.00—37 C.F.R. § 1.16(f))
Filing Fee Calculation $___________

C. [ ] Plant application
($480.00—37 C.F.R. § 1.16(g))
Filing Fee Calculation $___________

11. Small Entity Statement(s)

[ ] Statement(s) that this is a filing by a small entity under 37 C.F.R. §§ 1.9 and 1.27 is (are) attached.

WARNING: *"Status as a small entity must be specifically established in each application or patent in which the status is available and desired. Status as a small entity in one application or patent does not affect any other application or patent, including applications or patents which are directly or indirectly dependent upon the application or patent in which the status has been established. The refiling of an application under § 1.53 as a continuation, division, or continuation-in-part (including a continued prosecution application under § 1.53(d)), or the filing of a reissue application requires a new determination as to continued entitlement to small entity status for the continuing or reissue application. A nonprovisional application claiming benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) of a prior application, or a reissue application may rely on a statement filed in the prior application or in the patent if the nonprovisional application or the reissue application includes a reference to the statement in the prior application or in the patent or includes a copy of the statement in the prior application or in the patent and status as a small entity is still proper and desired. The payment of the small entity basic statutory filing fee will be treated as such a reference for purposes of this section." 37 C.F.R. § 1.28(a)(2).*

(complete the following, if applicable)

[] Status as a small entity was claimed in prior application _______/_____________, filed on ___________________ from which benefit is being claimed for this application under:

35 U.S.C. § [ ] 119(e),
[ ] 120,
[ ] 121,
[ ] 365(c),

and which status as a small entity is still proper and desired.

[ ] A copy of the statement in the prior application is included.

Filing Fee Calculation (50% of **A, B** or **C** above) $____________

NOTE: Any excess of the full fee paid will be refunded if a small entity status is established refund request are filed within 2 months of the date of timely payment of a full fee. The two-month period is not extendable under § 1.136. 37 C.F.R. § 1.28(a).

12. Request for International-Type Search (37 C.F.R. § 1.104(d))

(complete, if applicable)

[ ] Please prepare an international-type search report for this application at the time when national examination on the merits takes place.

13. Fee Payment Being Made at This Time

[X] Not Enclosed

[ ] No filing fee is to be paid at this time.
(This and the surcharge required by 37 C.F.R. § 1.16(e) can be paid subsequently.)

[ ] Enclosed

[ ] Filing fee $____________

[ ] Recording assignment
($40.00; 37 C.F.R. § 1.21(h))
(See attached "COVER SHEET FOR ASSIGNMENT ACCOMPANYING NEW APPLICATION.") $____________

[ ] Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached
($130.00; 37 C.F.R. §§ 1.47 and 1.17(i)) $____________

[ ] For processing an application with a specification in a non-English language
($130.00; 37 C.F.R. §§ 1.52(d) and 1.17(k)) $____________

[ ] Processing and retention fee
($130.00; 37 C.F.R. §§ 1.53(d) and 1.21(l)) $____________

[ ] Fee for international-type search report
($40.00; 37 C.F.R. § 1.21(e)) $___________

NOTE: 37 C.F.R. § 1.21(l) establishes a fee for processing and retaining any application that is abandoned for failing to complete the application pursuant to 37 C.F.R. § 1.53(f) and this, as well as the changes to 37 C.F.R. § 1.53 and 1.78(a)(1), indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid, or the processing and retention fee of § 1.21(l) must be paid, within 1 year from notification under § 53(f).

Total Fees Enclosed $___________

14. Method of Payment of Fees

[ ] Check in the amount of $___________.

[ ] Charge Account No. _________ in the amount of $__________.
A duplicate of this transmittal is attached.

NOTE: Fees should be itemized in such a manner that it is clear for which purpose the fees are paid. 37 C.F.R. § 1.22(b).

15. Authorization to Charge Additional Fees

WARNING: *If no fees are to be paid on filing, the following items should not be completed.*

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges, if extra claim charges are authorized.*

[ ] The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No.______.

[ ] 37 C.F.R. § 1.16(a), (f) or (g) (filing fees)

[ ] 37 C.F.R. § 1.16(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

[ ] 37 C.F.R. § 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)

[ ] 37 C.F.R. § 1.17(a)(1)-(5) (extension fees pursuant to § 1.136(a).

[ ] 37 C.F.R. § 1.17 (application processing fees)

NOTE: "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for

extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

[ ] 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b)).

NOTE: 37 C.F.R. § 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying, . . issue fee." From the wording of 37 C.F.R. § 1.28(b), (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

16. Instructions as to Overpayment

NOTE: ". . . Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

[ ] Credit Account No. ______.

[ ] Refund

SIGNATURE OF PRACTITIONER

Reg. No. 34,235
Tel. No.: (617) 345-6073

David S. Resnick
NIXON PEABODY LLP
101 Federal Street
Boston, MA 02110

Customer No.:

[X] **Incorporation by reference of added pages**

(check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED)

[X] Plus Added Pages for New Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed

Number of pages added 5

[ ] Plus Added Pages for Papers Referred to in Item 4 Above

Number of pages added ____________

[ ] Plus added pages deleting names of inventor(s) named on prior application(s) who is/are no longer inventor(s) of the subject matter claimed in this application.

Number of pages added ____________

[ ] Plus "Assignment Cover Letter Accompanying New Application"

Number of pages added ____________

[ ] **Statement Where No Further Pages Added**

(if no further pages form a part of this Transmittal, then end this Transmittal with this page and check the following item)

[ ] This transmittal ends with this page.

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ADDED PAGES FOR APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED

NOTE: See 37 C.F.R. § 1.78.

17. Relate Back

WARNING: *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. 120, 121 or 365(c). (35 U.S.C. 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

(complete the following, if applicable)

[X]Amend the specification by inserting, before the first line, the following sentence:

A. 35 U.S.C. 119(e)

NOTE: "Any nonprovisional application claiming the benefit of one or more prior filed copending provisional applications must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior provisional application, identifying it as a provisional application, and including the provisional application number (consisting of series code and serial number)." 37 C.F.R. § 1.78(a)(4).

[ ] "This application claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).: **FILING DATE**

_______/_______________________ _______________________"
_______/_______________________ _______________________"
_______/_______________________ _______________________"

B. 35 U.S.C. 120, 121 and 365(c)

NOTE: "Except for a continued prosecution application filed under § 1.53(d), any nonprovisional application claiming the benefit of one or more prior filed copending nonprovisional applications or international applications designating the United States of America must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior application, identifying it by application number (consisting of the series code and serial number) or international application number and international filing date and indicating the relationship of the applications. . . . Cross-references to other related applications may be made when appropriate." (See § 1.14(a)). 37 C.F.R. § 1.78(a)(2).

[X]"This application is a

[X]continuation

[ ] continuation-in-part

[ ] divisional

of copending application(s)

[ ] application number 0 /____________________ filed on ____________________"

[X] International Application PCT/US98/ 26114 filed on 9 December 1998 and which designated the U.S."

NOTE: The proper reference to a prior filed PCT application that entered the U.S. national phase is the U.S. serial number and the filing date of the PCT application that designated the U.S.

NOTE: (1) Where the application being transmitted adds subject matter to the International Application, then the filing can be as a continuation-in-part or (2) if it is desired to do so for other reasons then the filing can be as a continuation.

NOTE: The deadline for entering the national phase in the U.S. for an international application was clarified in the Notice of April 28, 1987 (1079 O.G. 32 to 46) as follows:

"The Patent and Trademark Office considers the International application to be pending until the 22nd month from the priority date if the United States has been designated and no Demand for International Preliminary Examination has been filed prior to the expiration of the 19th month from the priority date and until the 32nd month from the priority date if a Demand for International Preliminary Examination which elected the United States of America has been filed prior to the expiration of the 19th month from the priority date, provided that a copy of the international application has been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively. If a copy of the international application has not been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively, the international application becomes abandoned as to the United States 20 or 30 months from the priority date respectively. These periods have been placed in the rules as paragraph (h) of § 1.494 and paragraph (i) of § 1.495. A continuing application under 35 U.S.C. 365(c) and 120 may be filed anytime during the pendency of the international application."

[X] "The nonprovisional application designated above, namely application PCT / US98/26114, filed 9 December 1998, claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	**FILING DATE**
60 / 069,155	9 December 1997 "
60 / 069,687	12 December 1997 "
60 / 078,541	19 March 1998 "

[X]Where more than one reference is made above please combine all references into one sentence.

18. Relate Back—35 U.S.C. 119 Priority Claim for Prior Application

The prior U.S. application(s), including any prior International Application designating the U.S., identified above in item 17B, in turn itself claim(s) foreign priority(ies) as follows:

____

Country Appln. no. Filed

The certified copy(ies) has (have)

[ ] been filed on ____________________, in prior application 0 /______________, which was filed on ____________________.

[ ] is (are) attached.

WARNING: *The certified copy of the priority application that may have been communicated to the PTO by the International Bureau may not be relied on without any need to file a certified copy of the priority application in the continuing application. This is so because the certified copy of the priority application communicated by the International Bureau is placed in a folder and is not assigned a U.S. serial number unless the national stage is entered. Such folders are disposed of if the national stage is not entered. Therefore, such certified copies may not be available if needed later in the prosecution of a continuing application. An alternative would be to physically remove the priority documents from the folders and transfer them to the continuing application. The resources required to request transfer, retrieve the folders, make suitable record notations, transfer the certified copies, enter and make a record of such copies in the Continuing Application are substantial. Accordingly, the priority documents in folders of international applications that have not entered the national stage may not be relied on. Notice of April 28, 1987 (1079 O.G. 32 to 46).*

19. Maintenance of Copendency of Prior Application

NOTE: *The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the continuation application. Notice of November 5, 1985 (1060 O.G. 27).*

A. [ ] Extension of time in prior application

*(This item **must** be completed and the papers filed **in the prior application,** if the period set in the prior application has run.)*

[ ] A petition, fee and response extends the term in the pending **prior** application until ____________________

[ ] A **copy** of the petition filed in prior application is attached.

B. [ ] Conditional Petition for Extension of Time in Prior Application

(complete this item, if previous item not applicable)

[ ] A conditional petition for extension of time is being filed in the pending **prior** application.

[ ] **A copy** of the conditional petition filed in the prior application is attached.

20. Further Inventorship Statement Where Benefit of Prior Application(s) Claimed

(complete applicable item (a), (b) and/or (c) below)

(a) [ ] This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventor(s) in this application are

[ ] the same.

[ ] less than those named in the prior application. It is requested that the following inventor(s) identified for the prior application be deleted:

____

(type name(s) of inventor(s) to be deleted)

(b) [ ] This application discloses and claims additional disclosure by amendment and a new declaration or oath is being filed. With respect to the prior application, the inventor(s) in this application are

[ ] the same.

[ ] the following additional inventor(s) have been added:

____

(type name(s) of inventor(s) to be deleted)

(c) [ ] The inventorship for all the claims in this application are

[ ] the same.

[ ] not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made

[ ] is submitted.

[ ] will be submitted.

21. Abandonment of Prior Application *(if applicable)*

[ ] Please abandon the prior application at a time while the prior application is pending, or when the petition for extension of time or to revive in that application is granted, and when this application is granted a filing date, so as to make this application copending with said prior application.

NOTE: *According to the Notice of May 13, 1983 (103, TMOG 6-7), the filing of a continuation or continuation-in- part application is a proper response with respect to a petition for extension of time or a petition to revive and should include the express abandonment of the prior application conditioned upon the granting of the petition and the granting of a filing date to the continuing application.*

22. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment

WARNING: *"The claims of a new application may be finally rejected in the first Office action in those situations where (1) the new application is a continuing application of, or a substitute for, an earlier application, and (2) all the claims of the new application (a) are drawn to the same invention claimed in the earlier application, and (b) would have been properly finally rejected on the grounds of art of record in the next Office action if they had been entered in the earlier application." MPEP, § 706.07(b), 6th ed., rev.2.*

NOTE: *Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary.*

(check the next item, if applicable)

[ ] There is provided herewith a Petition To Suspend Prosecution for the Time Necessary to File An Amendment (New Application Filed Concurrently)

23. Small Entity (37 CFR § 1.28(a))

[ ] Applicant has established small entity status by the filing of a statement in parent application /____________ on ____________.

[ ] A copy of the statement previously filed is included.

WARNING: *See 37 CFR § 1.28(a).*

24. NOTIFICATION IN PARENT APPLICATION OF THIS FILING

[ ] A notification of the filing of this
(check one of the following)

[ ] continuation

[ ] continuation-in-part

[ ] divisional

is being filed in the parent application, from which this application claims priority under 35 U.S.C. § 120.

Practitioner's Docket No. 701039/48802 C

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Michael KLAGSBRUN, Shay SOKER, Hua-Quan MIAO, and Seiji TAKASHIMA
Application No.:
Filed: Herewith
For: ANTAGONISTS OF NEUROPILIN RECEPTOR FUNCTION AND USE THEREOF

Assistant Commissioner for Patents
Washington, D.C. 20231

EXPRESS MAIL CERTIFICATE

"Express Mail" label number EK571074376US
Date of Deposit 05/30/2000

I hereby state that the following *attached* paper or fee

1. New Application Transmittal (12 pages)
2. Added Pages for Application Transmittal where Benefit of Prior US Applications Claimed (5 pages)
3. Specification (46 pages)
4. Claims (2 pages)
5. Drawings (21 pages)
6. Abstract (1 page)
7. Sequence Listing (9 pages)

is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10, on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Nicholas A. Zachariades

Signature of person mailing paper or fee

Docket No. 1039/ 48802
Express Mail Label:

ANTAGONISTS OF NEUROPILIN RECEPTOR FUNCTION AND USE THEREOF

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

The work described herein was supported, in part, by National Institute of Health grants CA37392 and CA45548. The U.S. Government has certain rights to the invention.

FIELD OF THE INVENTION

The present invention relates to antagonists of neuropilin receptor function and use thereof in the treatment of cancer, particularly metastatic cancer, and angiogenic diseases.

BACKGROUND OF THE INVENTION

Cancer, its development and treatment is a major health concern. The standard treatments available are few and directed to specific types of cancer, and provide no absolute guarantee of success. Most treatments rely on an approach that involves killing off rapidly growing cells in the hope that rapidly growing cancerous cells will succumb, either to the treatment, or at least be sufficiently reduced in numbers to allow the body's system to eliminate the remainder. However most, of these treatments are non-specific to cancer cells and adversely effect non-malignant cells. Many cancers although having some phenotype relationship are quite diverse. Yet, what treatment works most effectively for one cancer may not be the best means for treating another cancer. Consequently, an appreciation of the severity of the condition must be made before beginning many therapies. In order to most effective, these treatments require not only an early detection of the malignancy, but an appreciation of the severity of the malignancy. Currently, it can be difficult to distinguish cells at a molecular level as it relates to effect on treatment. Thus, methods of being able to screen malignant cells and better understand their disease state are desirable.

While different forms of cancer have different properties, one factor which many cancers share is that they can metastasize. Until such time as metastasis occurs, a tumor, although it may be malignant, is confined to one area of the body. This may cause discomfort and/or pain, or even lead to more serious problems including death, but if it can be located, it may be surgically removed and, if done with adequate care, be treatable. However, once metastasis sets in, cancerous cells have invaded the body and while surgical resection may remove the parent tumor, this does not address other tumors. Only chemotherapy, or some particular form of targeting therapy, then stands any chance of success.

The process of tumor metastasis is a multistage event involving local invasion and destruction of intercellular matrix, intravasation into blood vessels, lymphatics or other channels of transport, survival in the circulation, extravasation out of the vessels in the secondary site and growth in the new location (Fidler, et al., *Adv. Cancer Res.* 28, 149-250 (1978), Liotta, et al., *Cancer Treatment Res.* 40, 223-238 (1988), Nicolson, *Biochim. Biophy. Acta* 948, 175-224 (1988) and Zetter, *N. Eng. J. Med.* 322, 605-612 (1990)). Success in establishing metastatic deposits requires tumor cells to be able to accomplish these steps sequentially. Common to many steps of the metastatic process is a requirement for motility. The enhanced movement of malignant tumor cells is a major contributor to the progression of the disease toward metastasis. Increased cell motility has been associated with enhanced metastatic potential in animal as well as human tumors (Hosaka, et al., *Gann* 69, 273-276 (1978) and Haemmerlin, et al., *Int. J. Cancer* 27, 603-610 (1981)).

Identifying factors that are associated with onset of tumor metastasis is extremely important. In addition, to using such factors for diagnosis and prognosis, those factors are an important site for identifying new compounds that can be used for treatment and as a target for treatment identifying new modes of treatment such as inhibition of metastasis is highly desirable.

Tumor angiogenesis is essential for both primary tumor expansion and metastatic tumor spread, and angiogenesis itself requires ECM degradation (Blood et al., *Biochim. Biophys. Acta* 1032:89-118 (1990)). Thus, malignancy is a systemic disease in which interactions between the neoplastic cells and their environment play a

crucial role during evolution of the pathological process (Fidler, I. *J., Cancer Metastasis Rev.* 5:29-49 (1986)).

There is mounting evidence that VEGF may be a major regulator of angiogenesis (reviewed in Ferrara, et al., *Endocr. Rev.*, 13, 18-32 (1992); Klagsbrun, et al., *Curr. Biol.*, 3, 699-702 (1993); Ferrara, et al., *Biochem. Biophjs. Res. Commun.*, 161, 851-858 (1989)). VEGF was initially purified from the conditioned media of folliculostellate cells (Ferrara, et al., *Biochem. Biophjs. Res. Commun.*, 161, 851-858 (1989)) and from a variety of tumor cell lines (Myoken, et al., *Proc. Natl. Acad. Sci.* USA, 88:5819-5823 (1991); Plouet, et al., *EMBO. J.*, 8:3801-3806 (1991)). VEGF was found to be identical to vascular permeability factor, a regulator of blood vessel permeability that was purified from the conditioned medium of U937 cells at the same time (Keck, et al., *Science*, 246:1309-1312 (1989)). VEGF is a specific mitogen for endothelial cells (EC) *in vitro* and a potent angiogenic factor *in vivo*. The expression of VEGF is up-regulated in tissue undergoing vascularization during embryogenesis and the female reproductive cycle (Brier, et al., *Development*, 114:521-532 (1992); Shweiki, et al., *J. Clin. Invest.*, 91:2235-2243 (1993)). High levels of VEGF are expressed in various types of tumors, but not in normal tissue, in response to tumor-induced hypoxia (Shweiki, et al., *Nature* 359:843-846 (1992); Dvorak et al., *J. Exp. Med.*, 174:1275-1278 (1991); Plate, et al., *Cancer Res.*, 53:5822-5827; Ikea, et al., *J. Biol. Chem.*, 270:19761-19766 (1986)). Treatment of tumors with monoclonal antibodies directed against VEGF resulted in a dramatic reduction in tumor mass due to the suppression of tumor angiogeneis (Kim, et al., *Nature*, 382:841-844 (1993)). VEGF appears to play a principle role in many pathological states and processes related to neovascularization. Regulation of VEGF expression in affected tissues could therefore be key in treatment or prevention of VEGF induced neovascularization/angiogenesis.

VEGF exists in a number of different isoforms that are produced by alternative splicing from a single gene containing eight exons (Ferrara, et al., *Endocr. Rev.*, 13:18-32 (1992); Tischer, et al., *J. Biol. Chem.*, 806:11947-11954 (1991); Ferrara, et al., *Trends Cardio Med.*, 3:244-250 (1993); Polterak, et al., *J. Biol. Chem.*, 272:7151-7158 (1997)). Human VEGF isoforms consists of monomers of 121, 145, 165, 189,

and 206 amino acids, each capable of making an active homodimer (Polterak et al., *J. Biol. Chem*, 272:7151-7158 (1997); Houck, et al., *Mol. Endocrinol.*, 8:1806-1814 (1991)). The $VEGF_{121}$ and $VEGF_{165}$ isoforms are the most abundant. $VEGF_{121}$ is the only VEGF isoforms that does not bind to heparin and is totally secreted into the culture medium. $VEGF_{165}$ is functionally different than $VEGF_{121}$ in that it binds to heparin and cell surface heparin sulfate proteoglycans (HSPGs) and is only partially released into the culture medium (Houck, et al., *J. Biol. Chem.*, 247:28031-28037 (1992); Park, et al., *Mol. Biol. Chem.*, 4:1317-1326 (1993)). The remaining isoforms are entirely associated with cell surface and extracellular matrix HSPGs (Houck, et al., *J. Biol. Chem.*, 247:28031-28037 (1992); Park, et al., *Mol. Biol. Chem.*, 4:1317-1326 (1993)).

VEGF receptor tyrosine kinases, KDR/Flk-1 and/or Flt-1, are mostly expressed by EC (Terman, et al., *Biochem. Biophys. Res. Commun.*, 187:1579-1586 (1992); Shibuya, et al., *Oncogene*, 5:519-524 (1990); De Vries, et al., *Science*, 265:989-991 (1992); Gitay-Goran, et al., *J. Biol. Chem.*, 287:6003-6096 (1992); Jakeman, et al., *J. Clin. Invest.*, 89:244-253 (1992)). It appears that VEGF activities such as mitogenicity, chemotaxis, and induction of morphological changes are mediated by KDR/Flk-1 but not Flt-1, even though both receptors undergo phosphorylation upon binding of VEGF (Millauer, et al., *Cell*, 72:835-846 (1993); Waltenberger, et al., *J. Biol. Chem.*, 269:26988-26995 (1994); Seetharam, et al., *Oncogene*, 10:135-147 (1995); Yoshida, et al., *Growth Factors*, 7:131-138 (1996)). Recently, Soker et al., identified a new VEGF receptor which is expressed on EC and various tumor-derived cell lines such as breast cancer-derived MDA-MB-231 (231) cells (Soker, et al., *J. Biol. Chem.*, 271:5761-5767 (1996)). This receptor requires the VEGF isoform to contain the portion encoded by exon 7. For example, although both $VEGF_{121}$ and $VEGF_{165}R$ bind to KDR/Flk-1 and Flt-1, only $VEGF_{165}$ binds to the new receptor. Thus, this is an isoform-specific receptor and has been named the $VEGF_{165}$ receptor ($VEGF_{165}R$). It will also bind the 189 and 206 isoforms. $VEGF_{165}R$ has a molecular mass of approximately 130 kDa, and it binds $VEGF_{165}$ with a Kd of about 2 X 10^{-10}M, compared with approximately 5 X 10^{-12}M for KDR/Flk-1. In structure-function analysis, it was shown directly that $VEGF_{165}$ binds to $VEGF_{165}R$ via its exon 7-

encoded domain which is absent in $VEGF_{121}$ (Soker, et al., *J. Biol. Chem.*, 271:5761-5767 (1996)). However, the function of the receptor was unclear.

Identifying the alterations in gene expression which are associated with malignant tumors, including those involved in tumor progression and angiogenesis, is clearly a prerequisite not only for a full understanding of cancer, but also to develop new rational therapies against cancer.

A further problem arises, in that the genes characteristic of cancerous cells are very often host genes being abnormally expressed. It is quite often the case that a particular protein marker for a given cancer while expressed in high levels in connection with that cancer is also expressed elsewhere throughout the body, albeit at reduced levels.

The current treatment of angiogenic diseases is inadequate. Agents which prevent continued angiogenesis, e.g, drugs (TNP-470), monoclonal antibodies, antisense nucleic acids and proteins (angiostatin and endostatin) are currently being tested. See, Battegay, *J. Mol. Med.*, 73, 333-346 (1995); Hanahan et al., *Cell*, 86, 353-364 (1996); Folkman, *N. Engl. J. Med.*, 333, 1757-1763 (1995). Although preliminary results with the antiangiogenic proteins are promising, there is still a need for identifying genes encoding ligands and receptors involved in angiogenesis for the development of new antiangiogenic therapies.

SUMMARY OF THE INVENTION

We have isolated a cDNA encoding the $VEGF_{165}$ R gene (SEQ ID NO: 1) and have deduced the amino acid sequence of the receptor (SEQ ID NO:2) We have discovered that this novel VEGF receptor is structurally unrelated to Flt-1 or KDR/Flk-1 and is expressed not only by endothelial cells but by non-endothelial cells, including surprisingly tumor cells.

In ascertaining the function of the $VEGF_{165}R$ we have further discovered that this receptor has been identified as a cell surface mediator of neuronal cell guidance and called neuropilin-1. Kolodkin et al., *Cell* 90:753-762 (1997). We refer to the receptor as $VEGF_{165}R$/NP-1 or NP-1.

In addition to the expression cloning of $VEGF_{165}$R/NP-1 cDNA we isolated another human cDNA clone whose predicted amino acid sequence was 47% homologous to that of $VEGF_{165}$R/NP-1 and over 90% homologous to rat neuropilin-2 (NP-2) which was recently cloned (Kolodkin, et al., *Cell 90*, 753-762 (1997)).

Our results indicate that $VEGF_{165}$R/NP-1 and NP-2 are expressed by both endothelial and tumor cells. (Fig. 19) We have shown that endothelial cells expressing both KDR and $VEGF_{165}$R/NP-1 respond with increased chemotaxis towards $VEGF_{165}$, not $VEGF_{121}$, when compared to endothelial cells expressing KDR alone. While not wishing to be bound by theory, we believe that $VEGF_{165}$R/NP-1 functions in endothelial cells to mediate cell motility as a co-receptor for KDR.

We have also shown in the Boyden chamber motility assay that $VEGF_{165}$ stimulates 231 breast carcinoma cell motility in a dose-response manner (Fig 15A). $VEGF_{121}$ had no effect motility of these cells (Fig 15B). Since tumor cells such as, 231 cells, do not express the VEGF receptors, KDR or Flt-1, while not wishing to be bound by theory, we believe that tumor cells are directly responsive to $VEGF_{165}$ via $VEGF_{165}$R/NP-1.

We have also analyzed two variants of Dunning rat prostate carcinoma cells, AT2.1 cells, which are of low motility and low metastatic potential, and AT3.1 cells, which are highly motile, and metastatic. Cross-linking and Northern blot analysis show that AT3.1 cells express abundant $VEGF_{165}$R/NP-1, capable of binding $VEGF_{165}$, while AT2.1 cells don't express $VEGF_{165}$R/NP-1 (Fig 18). Immunostaining of tumor sections confirmed the expression of $VEGF_{165}$R/NP-1 in AT3.1, but not AT2.1 tumors (Fig 17). Additionally, immunostaining showed that in subcutaneous AT3.1 and PC3 tumors, the tumor cells expressing $VEGF_{165}$R/NP-1 were found preferentially at the invading front of the tumor/dermis boundary (Fig 17). Furthermore, stable clones of AT2.1 cells overexpressing $VEGF_{165}$R/NP-1 had enhanced motility in the Boyden chamber assay. These results indicate that neuropilin expression on tumor cells is associated with the motile, metastatic phenotype and angiogenesis, and thus is an important target for antiangiogenic and anticancer therapy.

The present invention relates to antagonists of neuropilin (NP) receptor function that can be use to inhibit metastasis and angiogenesis. Antagonists of invention can block the receptor preventing ligand binding, disrupt receptor function, or inhibit receptor occurrence. Specific antagonists include, for example, compounds that bind to NP-1 or NP-2 and antibodies that specifically binds the receptor at a region that inhibits receptor function. For example, one can add an effective amount of a compound that binds to NP-1 to disrupt receptor fuction and thus inhibit metastasis.

We have surprisingly discovered that members of the semaphorin/collapsins family are not only inhibitors of neuronal guidance but also inhibitors of endothelial and tumor cell motility in cells that express neuropilin. Accordingly, preferred antagonists include members of the semaphorin/collapsins family or fragments thereof that bind NP and have VEGF antagonist activity as determined, for example, by the human umbilical vein endothelial cell (HUVEC) proliferation assay using $VEGF_{165}$ as set forth in Soker et al., *J. Biol. Chem. 272*, 31582-31588 (1997). Preferably, the semaphorin/collapsin has at least a 25% reduction in HUVEC proliferation, more preferably a 50% reduction, even more preferably a 75% reduction, most preferably a 95% reduction.

VEGF antagonist activity of the semaphorin/collapsin may also be determined by inhibition of binding of labeled $VEGF_{165}$ to $VEGF_{165}R$ as disclosed in Soker et al., *J. Biol. Chem. 271*, 5761-5767 (1996)) or to PAE/NP cells. Preferably, the portion inhibits binding by at least 25%, more preferably 50%, most preferably 75%.

In accordance with the present invention, neuropilin antagonists, or nucleic acids, e.g., DNA or RNA, encoding such antagonists, are useful as inhibitors of VEGF and NP function and can be used to treat diseases, disorders or conditions associated with VEGF and NP expression. The antagonists can be used alone or in combination with other anti-VEGF strategies including, for example, those that antagonize VEGF directly (e.g. anti-VEGF antibodies, soluble VEGF receptor extracellular domains), or antagonize VEGF receptors (e.g. anti-KDR antibodies, KDR kinase inhibitors, dominant-negative VEGF receptors) (Presta LG, et al., Cancer Res. 57: 4593-4599 (1997), Kendall RL, et al., (1996) Biochem. Biophys. Res. Commun. 226: 324-328,

Goldman CK, et al., (1998) Proc. Natl. Acad. Sci. USA 95: 8795-8800, Strawn LM, et al., (1996) Cancer Res. 56: 3540-3545, Zhu Z, et al., (1998). Cancer Res. 58: 3209-3214, Witte L, et al., (1998). Cancer Metastasis Rev. 17: 155-161.)

Diseases, disorders, or conditions, associated with VEGF, include, but are not limited to retinal neovascularization, hemagiomas, solid tumor growth, leukemia, metastasis, psoriasis, neovascular glaucoma, diabetic retinopathy, rheumatoid arthritis, endometriosis, mucular degeneration, osteoarthtitis, and retinopathy of prematurity (ROP).

In another embodiment, one can use isolated $VEGF_{165}R$/NP-1 or NP-2 or cells expressing these receptors in assays to discover compounds that bind to or otherwise interact with these receptors in order to discover NP antagonists that can be used to inhibit metastasis and/or angiogenesis.

Other aspects of the invention are disclosed *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows purification of $VEGF_{165}R$ From 231 Cells. ^{125}I-$VEGF_{165}$ (5 ng/ml) was bound and cross-linked to receptors on 231 cells and analyzed by SDS PAGE and autoradiography (lane 1). $VEGF_{165}R$ was purified by Con A and $VEGF_{165}$ affinity column chromatography and analyzed by SDS-PAGE and silver stain (lane 2). Two prominent bands were detected (arrows) and N-terminally sequenced separately. Their N-terminal 18 amino acid sequences are shown to the right of the arrows. The published N-terminal sequences of human and mouse neuropilin (Kawakami et al., *J. Neurobiol.*, *29*, 1-17 (1995); He and Tessier-Lavigne, *Cell* *90*, 739-751 1997) are shown below (SEQ ID NOS: 5 and 6).

Figures 2A and 2B show isolation of $VEGF_{165}R$ cDNA by Expression Cloning. Photomicrographs (dark field illumination) of COS 7 cells binding ^{125}I-$VEGF_{165}$. ^{125}I-$VEGF_{165}$ was bound to transfected COS 7 cells which were then washed, fixed, and overlayed with photographic emulsion that was developed as described in the example, *infra*.

Figure 2A shows COS 7 cells were transfected with a primary plasmid pool (#55 of the 231 cell library) representing approximately 3 x 10^3 clones and one COS 7 cell binding ^{125}I-VEGF$_{165}$ in the first round of screening is shown.

Figure 2 shows several COS 7 cells transfected with a single positive cDNA clone (A2) binding ^{125}I-VEGF$_{165}$ after the third round of screening.

Figure 3 shows the Deduced Amino Acid Sequence of Human VEGF$_{165}$R/NP-1 (SEQ ID NO:3). The deduced 923 amino acid sequence of the open reading frame of VEGF$_{165}$R/NP-1, clone A2 (full insert size of 6.5 kb) is shown. The putative signal peptide sequence (amino acids 1-21) and the putative transmembrane region (amino acids 860-883) are in boxes. The amino acid sequence obtained by N-terminal amino acid sequencing (Figure 3, amino acids 22-39) is underlined. The arrow indicates where the signal peptide has been cleaved and removed, based on comparison of the N-terminal sequence of purified VEGF$_{165}$R/NP-1 and the cDNA sequence. The sequence of human VEGF$_{165}$R/NP-1 reported here differs from that reported by He et al. (He and Tessier-Lavigne, *Cell 90*, 739-751 (1997)) in that we find Lys$_{26}$ rather than Glu$_{26}$, and Asp$_{855}$ rather than Glu$_{855}$ Lys$_{26}$ and Asp$_{855}$ are found, however, in mouse and rat VEGF$_{165}$R/NP-1 (Kwakami et al., *J. Neurobiol. 29*, 1-17 (1995); He and Tessier-Lavigne, *Cell 90*, 739-751 (1997)).

Figure 4 shows the Comparison of the Deduced Amino Acid Sequence of Human VEGF$_{165}$R/NP-1 (SEQ ID NO:2) and NP-2 (SEQ ID NO:4). The deduced open reading frame amino acid sequences of VEGF$_{165}$R/NP-1 and NP-2 are aligned using the DNASIS program. Amino acids that are identical in both open reading frames are shaded. The overall homology between the two sequences is 43%.

Figure 5 shows a Northern Blot Analysis of VEGF$_{165}$R/NP-1 Expression in Human EC and Tumor-Derived Cell Lines. Total RNA samples prepared from HUVEC (lane 1) and tumor-derived breast carcinoma, prostate carcinoma and melanoma cell lines as indicated (lanes 2-8) were resolved on a 1% agarose gel and blotted onto a GeneScreen nylon membrane. The membrane was probed with ^{32}P-

labeled $VEGF_{165}R$/NP-1 cDNA and exposed to X-ray film. Equal RNA loading was demonstrated by ethydium bromide staining of the gel prior to blotting. A major species of $VEGF_{165}R$/NP-1 mRNA of approximately 7 kb was detected in several of the cell lines.

Figure 6 shows a Northern Blot Analysis of $VEGF_{165}R$/NP-1 and KDR mRNA in Adult Human Tissues. A pre-made Northern blot membrane containing multiple samples of human mRNA (Clonetech) was probed with ^{32}P-labeled $VEGF_{165}R$/NP-1 cDNA (top) as described in Fig 5, and then stripped and reprobed with ^{32}P-labeled KDR cDNA (bottom).

Figures 7A and 7B show a Scatchard Analysis of $VEGF_{165}$ binding to $VEGF_{165}R$/NP-1. Figure 7A. Increasing amounts of ^{125}I-$VEGF_{165}$ (0.1-50 ng/ml) were added to subconfluent cultures of PAE cells transfected with human $VEGF_{165}R$/NP-1 cDNA (PAE/NP-1 cells) in 48 well dishes. Non-specific binding was determined by competition with a 200-fold excess of unlabeled $VEGF_{165}$. After binding, the cells were washed, lysed and the cell-associated radioactivity was determined using a γ counter.

Figure 7B. The binding data shown in Figure 7A were analyzed by the method of Scatchard, and a best fit plot was obtained with the LIGAND program (Munson and Rodbard, 1980). PAE/NP-1 cells express approximately 3×10^5 $VEGF_{165}$ binding sites per cell and bind ^{125}I-$VEGF_{165}$ with a K_d of 3.2×10^{-10} M.

Figure 8 shows cross-linking of $VEGF_{165}$ and $VEGF_{121}$ to PAE cells Expressing $VEGF_{165}R$/NP-1 and/or KDR. ^{125}I-$VEGF_{165}$ (5 ng/ml) (lanes 1-6) or ^{125}I-$VEGF_{121}$ (10 ng/ml) (lanes 7-10) were bound to subconfluent cultures of HUVEC (lane 1), PC3 (lane 2), PAE (lanes 3 and 7), a clone of PAE cells transfected with human $VEGF_{165}R$/NP-1 cDNA (PAE/NP-1) (lanes 4 and 8), a clone of PAE cells transfected with KDR (PAE/KDR) (lanes 5 and 9), and a clone of PAE/KDR cells transfected with human $VEGF_{165}R$/NP-1 cDNA (PAE/KDR/NP-1) (lanes 6 and 10).

The binding was carried out in the presence of 1 μg/ml heparin. At the end of a 2 hour incubation, each ^{125}I-VEGF isoform was chemically cross-linked to the cell surface. The cells were lysed and proteins were resolved by 6% SDS-PAGE. The polyacrylamide gel was dried and exposed to X-ray film. Solid arrows denote radiolabeled complexes containing ^{125}I-VEGF and KDR, open arrows denote radiolabeled complexes containing ^{125}I-VEGF and $VEGF_{165}$R/NP-1.

Figure 9 shows cross linking of $VEGF_{165}$ to PAE/KDR Cells Co-expressing $VEGF_{165}$R/NP-1 Transiently. PAE/KDR cells were transfected with pCPhygro or pCPhyg-NP-1 plasmids as described in "Experimental Procedures", and grown for 3 days in 6 cm dishes. ^{125}I-$VEGF_{165}$ (5 ng/ml) was bound and cross linked to parental PAE/KDR cells (lane 1), to PAE/KDR cells transfected with pCPhygro vector control (V) (lane 2), to PAE/KDR cells transfected with pCPhyg- $VEGF_{165}$R/NP-1 plasmids ($VEGF_{165}$R/NP-1) (lane 3), and to HUVEC (lane 4).). The binding was carried out in the presence of 1 μg/ml heparin. The cells were lysed and proteins were resolved by 6% SDS-PAGE as in Figure 8. Solid arrows denote radiolabeled complexes containing ^{125}I-$VEGF_{165}$ and KDR. Open arrows denote radiolabeled complexes containing ^{125}I-$VEGF_{165}$ and $VEGF_{165}$R/NP-1.

Figure 10 shows inhibition of ^{125}I-$VEGF_{165}$ binding to $VEGF_{165}$R/NP-1 interferes with its binding to KDR. ^{125}I-$VEGF_{165}$ (5 ng/ml) was bound to subconfluent cultures of PAE transfected with human $VEGF_{165}$R/NP-1 cDNA (PAE/NP-1) (lanes 1 and 2), PAE/KDR cells (lanes 3 and 4), and PAE/KDR cells transfected with human $VEGF_{165}$R/NP-1 cDNA (PAE/KDR/NP-1) (lanes 5 and 16) in 35 mm dishes. The binding was carried out in the presence (lanes 2, 4, and 6) or the absence (lanes 1, 3, and 5) of 25 μg/ml GST-Ex 7+8. Heparin (1 μg/ml) was added to each dish. At the end of a 2 hour incubation, ^{125}I-$VEGF_{165}$ was chemically cross-linked to the cell surface. The cells were lysed and proteins were resolved by 6% SDS-PAGE as in Figure 9. Solid arrows denote radiolabeled complexes containing

^{125}I-VEGF$_{165}$ and KDR, open arrows denote radiolabeled complexes containing ^{125}I-VEGF$_{165}$ and VEGF$_{165}$R/NP-1.

Figures 11A-C show a model for VEGF$_{165}$R/NP-1 modulation of VEGF$_{165}$ Binding to KDR. 11A.Cells expressing KDR alone. 11B.Cells co-expressing KDR and VEGF$_{165}$R/NP-1. 11C.Cells co-expressing KDR and VEGF$_{165}$R/NP-1 in the presence of GST- Ex 7+8 fusion protein.

A single KDR receptor or a KDR-VEGF$_{165}$R/NP-1 pair is shown in top portion. An expanded view showing several receptors is shown in the bottom portion. VEGF$_{165}$ binds to KDR via exon 4 and to VEGF$_{165}$R/NP-1 via exon 7 (Keyt et al. *J. Biol. Chem. 271*,5638-5646 (1996b); Soker et al., *J. Biol. Chem. 271*, 5761-5767 (1996)). A rectangular VEGF$_{165}$ molecule represents a suboptimal conformation that doesn't bind to KDR efficiently while a rounded VEGF$_{165}$ molecule represents one that fits better into a binding site. In cells expressing KDR alone, VEGF$_{165}$ binds to KDR in a sub-optimal manner. In cells co-expressing KDR and VEGF$_{165}$R/NP-1, the binding efficiency of VEGF$_{165}$ to KDR is enhanced. It may be that the presence of VEGF$_{165}$R/NP-1 increases the concentration of VEGF$_{165}$ on the cell surface, thereby presenting more growth factor to KDR. Alternatively, VEGF$_{165}$R/NP-1 may induce a change in VEGF$_{165}$ conformation that allows better binding to KDR, or both might occur. In the presence of GST-Ex 7+8, VEGF$_{165}$ binding to VEGF$_{165}$R/NP-1 is competitively inhibited and its binding to KDR reverts to a sub-optimal manner.

Figure 12 shows the human NP-2 amino acid sequence (SEQ ID NO:4).

Figures 13A and 13B show the human NP-2 DNA sequence (SEQ ID NO:3).

Figures 14A, 14B and 14C show the nucleotide (SEQ ID NO:1) and amino acid sequences (SEQ ID NO:2) of VEGF$_{165}$R/NP-1.

Figures 15A and 15B show $VEGF_{165}$ stimulation of MDA MB 231 cell motility. (Figure 15A) Dose response of $VEGF_{165}$ motility activity. (Figure 15B) Both $VEGF_{165}$ and bFGF stimulate motility but $VEGF_{121}$ does not.

Figures 16A, 16B and 16C show motility and neuropilin-1 expression of Dunning rat prostate carcinoma cell lines AT3-1 (high motility, high metastatic potential) and AT2.1 (low motility, low metastatic potential) cells. (Figure 16A) AT3.1 cells are more motile than AT2.1 cells in a Boyden chamber assay. 125I-$VEGF_{165}$ cross-links neuropilin-1 on AT3.1 cells but does not cross-link to AT2.1 cells. (Figure 16C) AT3.1 but not AT2.1 cells express neuropilin-1, while both cell types express VEGF.

Figures 17A, 17B and 17C show immunostaining of (Figure 17A) a PC3 subcutaneous tumor in a nude mouse, (Figure 17B) an AT3.1 tumor in a rat, (Figure 17C) an AT2.1 tumor in rat with anti-neuropilin-1 antibodies. Neuropilin immunostaining is preferentially associated with PC3 and AT3.1 tumor cells at the tumor/dermis boundary. Some of these cells cluster around blood vessels. AT2.1 cells do not express neuropilin-1.

Figures 18A and 18B show overexpression of neuropilin-1 in AT2.1 cells. (Figure 18A) Western blot, (Figure 18B) motility activity. Three AT2.1 clones (lanes 4,5,6) express higher amounts of neuropilin-1 protein and are more motile compared to parental AT2.1 cells or AT2.1 vector (AT2.1/V) controls and approach AT3.1 cell neuropilin-1 levels and migration activity.

Figure 19 shows expression of NP-1, NP-2 and β-actin in cancer cell lines and endothelial cells using reverse transcriptase PCR following primers:

Human NP-1
Forward (328-351): 5' TTTCGCAACGATAAATGTGGCGAT 3' (SEQ ID NO:7)
Reverse (738-719): 5' TATCACTCCACTAGGTGTTG 3' (SEQ ID NO:8)
Human NP-2
Forward (513-532): 5' CCAACCAGAAGATTGTCCTC 3' (SEQ ID NO:9)
Reverse (1181-1162): 5' GTAGGTAGATGAGGCACTGA 3'. (SEQ ID NO:10)

Figure 20 shows the effects of collapsin-1 treatment on PAE cell motility in a Boyden chamber. Collpasin-1 inhibits, by about 65% the basal migration of PAE cells expressing neuropilin-1 but not PAE cells expressing KDR. alone One collapsin unit is about 3 ng/ml.

Figures 21A and 21B show results of the aortic ring assay. Collapsin was added (Fig. 21A) or not added (Fig. 21B) to a segment of rat aortic ring and the migration of endothelial cells out of the rings and their formation of tubes was monitored after a week in organ culture. Migration and tube formation are inhibited by collapsin-1.

DETAILED DESCRIPTION OF THE INVENTION

We have discovered that there are VEGF receptors (VEGFR) and neuropilins such as $VEGF_{165}R$/NP-1 and NP-2 that are associated with metastatic potential of a malignant cell and angiogenesis. As used herein, "neuropilin" includes not only $VEGF_{165}R$/NP-1 and NP-2 but any neuropilin or VEGFR, where the constituents share at least about 85% homology with either of the above $VEGF_{165}R$/NP-1 and NP-2 can be used. More preferably, such constituent shares at least 90% homology. Still more preferably, each constituent shares at least 95% homology.

Homology is measured by means well known in the art. For example % homology can be determined by any standard algorithm used to compare homologies. These include, but are not limited to BLAST 2.0 such as BLAST 2.0.4 and i. 2.0.5 available from the NIH (See www.ncbi.nlm.nkh.gov/BLAST.newblast.html) (Altschul, S.F., et al. Nucleic Acids Res. 25: 3389-3402 (1997))and DNASIS (Hitachi Software Engineering America, Ltd.). These programs should preferably be set to an automatic setting such as the standard default setting for homology comparisons. As explained by the NIH, the scoring of gapped results tends to be more biologically meaningful than ungapped results.

For ease of reference, this disclosure will generally talk about $VEGF_{165}R$/NP-1 and NP-2 and/or homologs thereof but all teaching are applicable to the above-described homologs.

In another embodiment a VEGFR can be used as long as it binds to a sequence having at least 90%, more preferably 95% homology to exon 7 of $VEGF_{165}$. These VEGF receptors and neuropilins, e.g., $VEGF_{165}R$/NP-1 and NP-2, are associated with both tumor metastases and angiogenesis. We have shown that expression of $VEGF_{165}R$/NP-1 and NP-2 is upregulated in highly metastatic prostate cancer cell lines relative to poorly metastatic or nonmetastatic lines. Thus, expression of $VEGF_{165}R$/NP-1 and NP-2 is associated with a tumors metastatic potential.

In accordance with the present invention, antagonists of neuropilin receptor function can be used inhibit or prevent the metastasis process and/or angiogenesis. Antagonists of the invention can block the receptors preventing ligand binding, disrupt receptor function, or inhibit receptor occurrence. Specific antagonists include, for example, compounds that bind to NP-1 or NP-2 and antibodies that specifically binds the receptor at a region that inhibits receptor function. For example, one can add an effective amount of a compound that binds to NP-1 to disrupt receptor fuction and thus inhibit metastasis.

Preferred antagonists include members of the semaphorin/collapsins family. We have surprisingly discovered that members of the semaphorin/collapsins family are not only inhibitors of neuronal guidance but also inhibitors of endothelial and tumor cell motility in cells that express neuropilin. Collapsin-1 is a particularly preferred antagonist. Other members of the semaphorin collapsin family can be selected by screening for neuropilin binding.

Semaphorin/collapsins are a family of 100 kDa glycoproteins (Luo, et al. (1993) *Cell* 75: 217-2271 Kolodkin, et al., (1993) *Cell* 75: 1389-1399, Behar, et al., (1996) *Nature* 383: 525-528.)Semaphorins are the mammalian homologue and collapsins are the chick homologue. Semaphorins are expressed primarily in the developing CNS, but are also found in developing bones and heart. The receptors for the semaphorins are neuropilin-1 and neuropilin-2 (He, et al., *Cell* 90, 739-751 (1997), Kolodkin, et al, *Cell* 90, 753-762 (1997)) and there is ligand binding

specificity for different semaphorin family members (Chen, et al., *Neuron* 19:547-559 (1997)). The K_d for semaphorin binding is about 3 x 10^{-10}M, similar to that for $VEGF_{165}$ binding to neuropilin-1. Semaphorins mediate neuronal guidance by repelling and collapsing advancing dorsal root ganglion (DRG) growth cones.

Semaphorin/collapsins are know in the art and can be isolated from natural sources or produced using recombinant DNA methods. See, for example, U.S. Patent 5,807,826. Additionally, fragments of the semaphorin/collapsins may be used. For example, a 70 amino acid region within the semaphorin domain specifies the biological activities of three collapsin family members (Koppel, et al., *Neuron* 19: 531-537).

Pure recombinant chick collapsin-1 (semaphorin III) was can be produced by the methods set forth in the following references (Luo, et al. (1993) *Cell* 75: 217-227.); Koppel, et al. *J. Biol. Chem.* 273: 15708-15713, Feiner, et al. (1997) *Neuron* 19: 539-545).

We have shown that when collapsin-1 was added to cultures of porcine endothelial cells (PAE) and PAE neuropilin-1 and/or KDR transfectants, ^{125}I-Collapsin was found to bind to PAE cells expressing neuropilin-1 but not to PAE cells expressing KDR. Furthermore, in a Boyden chamber assay, collapsin-1 inhibited the basal migration of PAE expressing neuropilin-1. by about 60-70%, but had no effect on parental PAE or PAE expressing KDR alone (Fig. 20). Inhibition was dose-dependent and half-maximal inhibition occurred with 50 collapsing units/ml (as measured on DRG, 1 CU = 3 ng/ml). Thus, semaphorin/collapsins inhibit the motility of non-neuronal cells as long as neuropilin-1 is expressed.

Antibodies that specifically binds the NP at a region that inhibits receptor function can also be used as antagonists of the invention. Antibodies may be raised against either a peptide of the receptor or the whole molecule. Such a peptide may be presented together with a carrier protein, such as an KLH, to an animal system or, if it is long enough, say 25 amino acid residues, without a carrier.

In accordance with yet another aspect of the present invention, there are provided isolated antibodies or antibody fragments which selectively binds the receptor. The antibody fragments include, for example, Fab, Fab', F(ab')2 or Fv

fragments. The antibody may be a single chain antibody, a humanized antibody or a chimeric antibody.

Antibodies, or their equivalents, or other receptor antagonists may also be used in accordance with the present invention for the treatment or prophylaxis of cancers. Administration of a suitable dose of the antibody or the antagonist may serve to block the receptor and this may provide a crucial time window in which to treat the malignant growth.

Prophylaxis may be appropriate even at very early stages of the disease, as it is not known what specific event actually triggers metastasis in any given case. Thus, administration of the antagonists which interfere with receptor activity, may be effected as soon as cancer is diagnosed, and treatment continued for as long as is necessary, preferably until the threat of the disease has been removed. Such treatment may also be used prophylactically in individuals at high risk for development of certain cancers, e.g., prostate or breast.

It will be appreciated that antibodies for use in accordance with the present invention may be monoclonal or polyclonal as appropriate. Antibody equivalents of these may comprise: the Fab' fragments of the antibodies, such as Fab, Fab', F(ab')2 and Fv; idiotopes; or the results of allotope grafting (where the recognition region of an animal antibody is grafted into the appropriate region of a human antibody to avoid an immune response in the patient), for example. Single chain antibodies may also be used. Other suitable modifications and/or agents will be apparent to those skilled in the art.

Chimeric and humanized antibodies are also within the scope of the invention. It is expected that chimeric and humanized antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody. A variety of approaches for making chimeric antibodies, comprising for example a non-human variable region and a human constant region, have been described. See, for example, Morrison et al., Proc. Natl. Acad. Sci. U.S.A. 81,6851 (1985); Takeda, et al., Nature 314,452(1985), Cabilly et al., U.S. Pat. No. 4,816,567; Boss et al., U.S. Pat. No. 4,816,397; Tanaguchi et al., European Patent Publication EP 171496; European Patent Publication 0173494, United Kingdom Patent GB 2177096B. Additionally, a chimeric antibody can be

further "humanized" such that parts of the variable regions, especially the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such altered immunoglobulin molecules may be made by any of several techniques known in the art, (e.g., Teng et al., Proc. Natl. Acad Sci. U.S.A., 80, 7308-7312 (1983); Kozbor et al., Immunology Today, 4, 7279 (1983); Olsson et al., Meth. Enzymol., 92, 3-16 (1982)), and are preferably made according to the teachings of PCT Publication WO92/06193 or EP 0239400. Humanized antibodies can be commercially produced by, for example, Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.

The present invention further provides use of neuropilin for intracellular or extracellular targets to affect binding. Intracellular targeting can be accomplished through the use of intracellularly expressed antibodies referred to as intrabodies. Extracellular targeting can be accomplished through the use of receptor specific antibodies.

These methods can be used to inhibit metastasis in malignant cells as we have found that the presence of these receptors is positively correlated with metastasis. One can treat a range of afflictions or diseases associated with expression of the receptor by directly blocking the receptor. This can be accomplished by a range of different approaches. One preferred approach is the use of antibodies that specifically block VEGF binding to the receptor. For example, an antibody to the VEGF binding site. Antibodies to these receptors can be prepared by standard means. For example, one can use single chain antibodies to target these binding sites.

The antibody can be administered by a number of methods. One preferred method is set forth by Marasco and Haseltine in PCT WO94/02610, which is incorporated herein by reference. This method discloses the intracellular delivery of a gene encoding the antibody. One would preferably use a gene encoding a single chain antibody. The antibody would preferably contain a nuclear localization sequence. One preferably uses an SV40 nuclear localization signal. By this method one can intracellularly express an antibody, which can block $VEGF_{165}R$/NP-1 or NP-2 functioning in desired cells.

DNA encoding human $VEGF_{165}R$/NP-1 or NP-2 and recombinant human $VEGF_{165}R$/NP-1 or NP-2 may be produced according to the methods set forth in the Examples.

The receptors are preferably produced by recombinant methods. A wide variety of molecular and biochemical methods are available for generating and expressing the polypeptides of the present invention; see e.g. the procedures disclosed in *Molecular Cloning, A Laboratory Manual* (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor), *Current Protocols in Molecular Biology* (Eds. Aufubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y. 1992) or other procedures that are otherwise known in the art. For example, the polypeptides of the invention may be obtained by chemical synthesis, expression in bacteria such as *E. coli* and eukaryotes such as yeast, baculovirus, or mammalian cell-based expression systems, etc., depending on the size, nature and quantity of the polypeptide.

The term "isolated" means that the polypeptide is removed from its original environment (e.g., the native VEGF molecule). For example, a naturally-occurring polynucleotides or polypeptides present in a living animal is not isolated, but the same polynucleotides or DNA or polypeptides, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

Where it is desired to express the receptor or a fragment thereof, any suitable system can be used. The general nature of suitable vectors, expression vectors and constructions therefor will be apparent to those skilled in the art.

Suitable expression vectors may be based on phages or plasmids, both of which are generally host-specific, although these can often be engineered for other hosts. Other suitable vectors include cosmids and retroviruses, and any other vehicles, which may or may not be specific for a given system. Control sequences, such as recognition, promoter, operator, inducer, terminator and other sequences essential

and/or useful in the regulation of expression, will be readily apparent to those skilled in the art.

Correct preparation of nucleotide sequences may be confirmed, for example, by the method of Sanger et al. (*Proc. Natl. Acad. Sci.* USA 74:5463-7 (1977)).

A DNA fragment encoding the receptor or fragment thereof, may readily be inserted into a suitable vector. Ideally, the receiving vector has suitable restriction sites for ease of insertion, but blunt-end ligation, for example, may also be used, although this may lead to uncertainty over reading frame and direction of insertion. In such an instance, it is a matter of course to test transformants for expression, 1 in 6 of which should have the correct reading frame. Suitable vectors may be selected as a matter of course by those skilled in the art according to the expression system desired.

By transforming a suitable organism or, preferably, eukaryotic cell line, such as HeLa, with the plasmid obtained, selecting the transformant with ampicillin or by other suitable means if required, and adding tryptophan or other suitable promoter-inducer (such as indoleacrylic acid) if necessary, the desired polypeptide or protein may be expressed. The extent of expression may be analyzed by SDS polyacrylamide gel electrophoresis-SDS-PAGE (Lemelli, *Nature* 227:680-685 (1970)).

Suitable methods for growing and transforming cultures etc. are usefully illustrated in, for example, Maniatis (Molecular Cloning, A Laboratory Notebook, Maniatis et al. (eds.), Cold Spring Harbor Labs, N.Y. (1989)).

Cultures useful for production of polypeptides or proteins may suitably be cultures of any living cells, and may vary from prokaryotic expression systems up to eukaryotic expression systems. One preferred prokaryotic system is that of *E. coli*, owing to its ease of manipulation. However, it is also possible to use a higher system, such as a mammalian cell line, for expression of a eukaryotic protein. Currently preferred cell lines for transient expression are the HeLa and Cos cell lines. Other expression systems include the Chinese Hamster Ovary (CHO) cell line and the baculovirus system.

Other expression systems which may be employed include streptomycetes, for example, and yeasts, such as Saccharomyces spp., especially S. cerevisiae. Any

system may be used as desired, generally depending on what is required by the operator. Suitable systems may also be used to amplify the genetic material, but it is generally convenient to use *E. coli* for this purpose when only proliferation of the DNA is required.

The polypeptides and proteins may be isolated from the fermentation or cell culture and purified using any of a variety of conventional methods including: liquid chromatography such as normal or reversed phase, using HPLC, FPLC and the like; affinity chromatography (such as with inorganic ligands or monoclonal antibodies); size exclusion chromatography; immobilized metal chelate chromatography; gel electrophoresis; and the like. One of skill in the art may select the most appropriate isolation and purification techniques without departing from the scope of this invention.

The present invention also provides binding assays using $VEGF_{165}R$/NP-1 or NP-2 that permit the ready screening for compounds which affect the binding of the receptor and its ligands, e.g., $VEGF_{165}$. These assays can be used to identify compounds that modulate, preferably inhibit metastasis and/or angiogenesis. However, it is also important to know if a compound enhances metastasis so that its use can be avoided. For example, in a direct binding assay the compound of interest can be added before or after the addition of the labeled ligand, e.g., $VEGF_{165}$, and the effect of the compound on binding or cell motility or angiogenesis can be determined by comparing the degree of binding in that situation against a base line standard with that ligand, not in the presence of the compound. The assay can be adapted depending upon precisely what is being tested.

The preferred technique for identifying molecules which bind to the neuropilin receptor utilizes a receptor attached to a solid phase, such as the well of an assay plate. The binding of the candidate molecules, which are optionally labeled (e.g., radiolabeled), to the immobilized receptor can be measured. Alternatively, competition for binding of a known, labeled receptor ligand, such as I-^{125} $VEGF_{165}$, can be measured. For screening for antagonists, the receptor can be exposed to a receptor ligand, e.g., $VEGF_{165}$, followed by the putative antagonist, or the ligand and antagonist can be added to the receptor simultaneously, and the ability of the

antagonist to block receptor activation can be evaluated. For example, VEGF antagonist activity may also be determined by inhibition of binding of labeled $VEGF_{165}$ to $VEGF_{165}R$ as disclosed in the Examples.

The ability of discovered antagonists to influence angiogenesis or metastasis can also be determined using a number of know *in vivo* and *in vitro* assays. Such assays are disclosed in Jain et al., *Nature Medicine* 3, 1203-1208(1997), and the examples.

Where the present invention provides for the administration of, for example, antibodies to a patient, then this may be by any suitable route. If the tumor is still thought to be, or diagnosed as, localized, then an appropriate method of administration may be by injection direct to the site. Administration may also be by injection, including subcutaneous, intramuscular, intravenous and intradermal injections.

Formulations may be any that are appropriate to the route of administration, and will be apparent to those skilled in the art. The formulations may contain a suitable carrier, such as saline, and may also comprise bulking agents, other medicinal preparations, adjuvants and any other suitable pharmaceutical ingredients. Catheters are one preferred mode of administration.

Neuropilin expression may also be inhibited *in vivo* by the use of antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. An antisense nucleic acid molecule which is complementary to a nucleic acid molecule encoding receptor can be designed based upon the isolated nucleic acid molecules encoding the receptor provided by the invention. An antisense nucleic acid molecule can comprise a nucleotide sequence which is complementary to a coding strand of a nucleic acid, e.g. complementary to an mRNA sequence, constructed according to the rules of Watson and Crick base pairing, and can hydrogen bond to the coding strand of the nucleic acid. The antisense sequence complementary to a sequence of an mRNA can be complementary to a sequence in the coding region of the mRNA or can be complementary to a 5' or 3' untranslated region of the mRNA. Furthermore, an antisense nucleic acid can be complementary in sequence to a regulatory region of the gene encoding the mRNA,

for instance a transcription initiation sequence or regulatory element. Preferably, an antisense nucleic acid complementary to a region preceding or spanning the initiation codon or in the 3' untranslated region of an mRNA is used. An antisense nucleic acid can be designed based upon the nucleotide sequence shown in SEQ ID NO:1 ($VEGF_{165}R$/NP-1) or SEQ ID NO:3 (NP-2). A nucleic acid is designed which has a sequence complementary to a sequence of the coding or untranslated region of the shown nucleic acid. Alternatively, an antisense nucleic acid can be designed based upon sequences of a $VEGF_{165}R$ gene, which can be identified by screening a genomic DNA library with an isolated nucleic acid of the invention. For example, the sequence of an important regulatory element can be determined by standard techniques and a sequence which is antisense to the regulatory element can be designed.

The antisense nucleic acids and oligonucleotides of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. The antisense nucleic acid or oligonucleotide can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids e.g. phosphorothioate derivatives and acridine substituted nucleotides can be used. Alternatively, the antisense nucleic acids and oligonucleotides can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e. nucleic acid transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest). The antisense expression vector is introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. et al., Antisense RNA as a molecular tool for genetic analysis, Reviews - Trends in Genetics, Vol. 1 (1)1986.

The term "pharmaceutically acceptable" refers to compounds and compositions which may be administered to mammals without undue toxicity. Exemplary pharmaceutically acceptable salts include mineral acid salts such as hydrochlorides,

hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like.

The antagonists of the invention are administered orally, topically, or by parenteral means, including subcutaneous and intramuscular injection, implantation of sustained release depots, intravenous injection, intranasal administration, and the like. Accordingly, antagonists of the invention may be administered as a pharmaceutical composition comprising the antibody or nucleic acid of the invention in combination with a pharmaceutically acceptable carrier. Such compositions may be aqueous solutions, emulsions, creams, ointments, suspensions, gels, liposomal suspensions, and the like. Suitable carriers (excipients) include water, saline, Ringer's solution, dextrose solution, and solutions of ethanol, glucose, sucrose, dextran, mannose, mannitol, sorbitol, polyethylene glycol (PEG), phosphate, acetate, gelatin, collagen, Carbopol Registered TM , vegetable oils, and the like. One may additionally include suitable preservatives, stabilizers, antioxidants, antimicrobials, and buffering agents, for example, BHA, BHT, citric acid, ascorbic acid, tetracycline, and the like. Cream or ointment bases useful in formulation include lanolin, Silvadene Registered TM (Marion), Aquaphor Registered TM (Duke Laboratories), and the like. Other topical formulations include aerosols, bandages, and other wound dressings. Alternatively one may incorporate or encapsulate the compounds such as an antagonist in a suitable polymer matrix or membrane, thus providing a sustained-release delivery device suitable for implantation near the site to be treated locally. Other devices include indwelling catheters and devices such as the Alzet Registered TM minipump. Ophthalmic preparations may be formulated using commercially available vehicles such as Sorbi-care Registered TM (Allergan), Neodecadron Registered TM (Merck, Sharp & Dohme), Lacrilube Registered TM , and the like, or may employ topical preparations such as that described in U.S. Pat. No. 5,124,155, incorporated herein by reference. Further, one may provide an antagonist in solid form, especially as a lyophilized powder. Lyophilized formulations typically contain stabilizing and bulking agents, for example human serum albumin, sucrose, mannitol, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co.).

The NP antagonists of the invention can be combined with a therapeutically effective amount of another molecule which negatively regulates angiogenesis which may be, but is not limited to, TNP-470, platelet factor 4, thrombospondin-1, tissue inhibitors of metalloproteases (TIMPl and TIMP2), prolactin (16-Kd fragment), angiostatin (38-Kd fragment of plasminogen), endostatin, bFGF soluble receptor, transforming growth factor beta, interferon alfa, soluble KDR and FLT-1 receptors and placental proliferin-related protein.

An NP antagonist of the invention may also be combined with chemotherapeutic agents.

The DNA encoding an antagonist, e.g., a collapsin, can be used in the form of gene therapy and delivered to a host by any method known to those of skill in the art to treat disorders associated with VEGF.

The amount of an NP antagonist required to treat any particular disorder will of course vary depending upon the nature and severity of the disorder, the age and condition of the subject, and other factors readily determined by one of ordinary skill in the art.

All references cited above or below are herein incorporated by reference.

The present invention is further illustrated by the following Examples. These Examples are provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLE 1

Experimental procedures
Materials

Cell culture media, lipofectin and lipofectamin reagents for transfection were purchased from Life Technologies. Human recombinant $VEGF_{165}$ and $VEGF_{121}$ were produced in Sf-21 insect cells infected with recombinant baculovirus vectors encoding either human $VEGF_{165}$ or $VEGF_{121}$ as previously described (Cohen et al., *Growth Factors*, 7, 131-138 (1992); Cohen et al., *J. Biol. Chem.*, 270, 11322-11326 (1995)). GST VEGF exons 7+8 fusion protein was prepared in E.Coli and purified as previously described (Soker et al., *J. Biol. Chem., 271*, 5761-5767 (1996)). Heparin,

hygromycin B and protease inhibitors were purchased from Sigma (St. Louis, MO). ^{125}I-Sodium, ^{32}P-dCTP, and GeneScreen-Plus hybridization transfer membrane were purchased from DuPont NEN (Boston, MA). Disuccinimidyl suberate (DSS) and IODO-BEADS were purchased from Pierce Chemical Co. (Rockford, IL). Con A Sepharose was purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). RNAzol-B was purchased from TEL-TEST Inc. (Friendswood, TX). Silver Stain kit and Trans-Blot PVDF membranes were purchased from Bio-Rad Laboratories (Hercules, CA). Multiple tissue northern blot membranes were purchased from Clontech (Palo Alto, CA). PolyATract mRNA isolation kits were purchased from Promega (Madison, WI). RediPrime DNA labeling kits and molecular weight markers were purchased from Amersham (Arlington Heights, IL). Plasmids: pcDNA3.1 was purchased from Invitrogen (Carlsbad, CA), and pCPhygro, containing the CMV promoter and encoding hygromycin B phosphorylase, was kindly provided by Dr. Urban Deutsch (Max Plank Institute, Bad Nauheim, Germany). Restriction endonucleases and Ligase were purchased from New England Biolabs, Inc (Beverly, MA). NT-B2 photographic emulsion and x-ray film were purchased from the Eastman Kodak company (Rochester NY).

Cell culture

Human umbilical vein EC (HUVEC) were obtained from American Type Culture Collection (ATCC) (Rockville, MD), and grown on gelatin coated dishes in M-199 medium containing 20% fetal calf serum (FCS) and a mixture of glutamine, penicillin and streptomycin (GPS). Basic FGF (2 ng/ml) was added to the culture medium every other day. Parental porcine aortic endothelial (PAE) cells and PAE cells expressing KDR (PAE/KDR) (Waltenberger et al., *J. Biol. Chem. 269*, 26988-26995 (1994)) were kindly provided by Dr. Lena Claesson-Welsh and were grown in F12 medium containing 10% FCS and GPS. MDA-MB-231 cells and MDA-MB-453 cells were obtained from ATCC, and grown in DMEM containing 10% FCS and GPS. The human melanoma cell lines, RU-mel, EP-mel and WK-mel were kindly provided by Dr. Randolf Byer (Boston University Medical School, Boston, MA), and grown in DMEM containing 2% FCS, 8% calf serum and GPS. Human metastatic prostate

adenocarcinoma, LNCaP and prostate carcinoma, PC3 cells were kindly provided by Dr. Michael Freeman (Children's Hospital, Boston, MA), and grown in RPMI 1640 containing 5% FCS and GPS.

Purification and protein sequencing

Approximately 5 x 10^8 MDA-MB-231 cells grown in 150 cm dishes were washed with PBS containing 5 mM EDTA, scraped and centrifuged for 5 min at 500g. The cell pellet was lysed with 150 ml of 20 mM HEPES, pH 8.0, 0.5% triton X-100 and protease inhibitors including 1 mM AEBSF, 5 μg/ml leupeptin and 5 μg/ml aprotinin for 30 min on ice, and the lysate was centrifuged at 30,000 x g for 30 min. $MnCl_2$ and $CaCl_2$ were added to the supernatant to obtain a final concentration of 1mM each. The lysate was absorbed onto a Con A Sepharose column (7 ml) and bound proteins were eluted with 15 ml 20 mM HEPES, pH 8.0, 0.2 M NaCl, 0.1% triton X-100 and 1 M methyl-α-D-mannopyranoside at 0.2 ml/min. The elution was repeated twice more at 30 minute intervals. The Con A column eluates were pooled and incubated for 12 h at 4° C with 0.5 ml of $VEGF_{165}$- Sepharose beads, containing about 150 μg $VEGF_{165}$, prepared as described previously (Wilchek and Miron, *Biochem. Int. 4*, 629-635. (1982)). The $VEGF_{165}$-Sepharose beads were washed with 50 ml of 20 mM HEPES, pH 8.0, 0.2 M NaCl and 0.1% triton X-100 and then with 25 ml of 20 mM HEPES, pH 8.0. The beads were boiled in SDS-PAGE buffer and bound proteins were separated by 6% SDS-PAGE. Proteins were transferred to a TransBlot PVDF membrane using a semi-dry electric blotter (Hoeffer Scientific), and the PVDF membrane was stained with 0.1% Coomassie Brilliant Blue in 40% methanol. The two prominent proteins in a 130-140 kDa doublet were cut out separately and N-terminally sequenced using an Applied Biosystems model 477A microsequenator as a service provided by Dr. William Lane of the Harvard Microchemistry facility (Cambridge, MA).

Expression cloning and DNA sequencing

Complementary DNA (cDNA) was synthesized from 5 μg 231 mRNA. Double-stranded cDNA was ligated to *EcoR*I adaptors, and size-fractionated on a 5-20% potassium acetate gradient . DNA fragments larger than 2kb were ligated to an eukaryotic expression plasmid, pcDNA3.1. The plasmid library was transfected into

E.coli to yield a primary library of approximately 1 x 10^7 individual clones. A portion of the transformed bacteria was divided into 240 pools, each representing approximately 3 x 10^3 individual clones. DNA prepared from each pool was used to transfect COS-7 cells seeded in 12 well dishes, using the Lipofectin reagent according to the manufacturer's instructions. Three days after transfection, the cells were incubated on ice for 2 h with ^{125}I-VEGF$_{165}$ (10 ng/ml) in the presence of 1 μg/ml heparin, washed and fixed with 4% paraformaldehyde in PBS. ^{125}I-VEGF$_{165}$ binding to individual cells was detected by overlaying the monolayers with photographic emulsion, NT-B2, and developing the emulsion after two days as described (Gearing et al.,1989). Seven positive DNA pools were identified and DNA from one of the positive pools was used to transform E.Coli . The E. coli were sub-divided into 50 separate pools and plated onto 50 LB ampicillin dishes, with each pool representing approximately 100 clones. DNA made from these pools was transfected into COS-7 cells which were screened for ^{125}I-VEGF$_{165}$ binding as described above. Twenty positive pools were detected at this step, and their corresponding DNAs were used to transform *E. Coli.* Each pool was plated onto separate LB ampicillin dishes and DNA was prepared from 96 individual colonies and screened in a 96-well two dimensional grid for ^{125}I-VEGF$_{165}$ binding to tranfected COS-7 cells as described above. Seven single clones were identified as being positive at this step. The seven positive plasmid clones were amplified and their DNA was analyzed by restriction enzyme digestion. Six clones showed an identical digestion pattern of digest and one was different. One clone from each group was submitted for automated DNA sequencing.

Northern Analysis

Total RNA was prepared from cells in culture using RNAzol according to the manufacturer's instructions. Samples of 20 μg RNA were separated on a 1% formaldehide-agarose gel, and transferred to a GeneScreen-Plus membrane. The membrane was hybridized with a ^{32}P labeled fragment of human VEGF$_{165}$R/NP-1 cDNA, corresponding to nucleotides 63-454 in the ORF, at 63°C for 18 h. The membrane was washed and exposed to an x-ray film for 18 h. A commercially-obtained multiple human adult tissue mRNA blot (Clonetech, 2 μg/lane) was probed

for human NP-1 in a similar manner. The multiple tissue blot was stripped by boiling in the presence of 0.5% SDS and re-probed with a ^{32}P labeled fragment of KDR cDNA corresponding to nucleotides 2841-3251 of the ORF (Terman et al., *Oncogene* 6, 1677-1683 (1991)).

Transfection of PAE cells

Parental PAE cells and PAE cells expressing KDR (PAE/KDR) (Waltenberger et al., 1994) were obtained from Dr. Lena Claesson-Welsh. Human NP-1 cDNA was digested with *Xho*I and *Xba*I restriction enzymes and subcloned into the corresponding sites of pCPhygro, to yield pCPhyg-NP-1. PAE and PAE/KDR cells were grown in 6 cm dishes and transfected with 5 μg of pCPhyg-NP-1 using Lipofectamine, according to the manufacturer's instructions. Cells were allowed to grow for an additional 48 h and the medium was replaced with fresh medium containing 200 μg/ml hygromycin B. After 2 weeks, isolated colonies (5-10 x 10^3 cell/colony) were transferred to separate wells of a 48 well dish and grown in the presence of 200 μg /ml hygromycin B. Stable PAE cell clones expressing $VEGF_{165}R$/NP-1 (PAE/NP-1) or co-expressing $VEGF_{165}R$/NP-1 and KDR (PAE/KDR/NP-1) were screened for $VEGF_{165}$ receptor expression by binding and cross linking of ^{125}I-$VEGF_{165}$. For transient transfection, PAE/KDR cells were transfected with $VEGF_{165}R$/NP-1 as described above and after three days ^{125}I-$VEGF_{165}$ cross-linking analysis was carried out.

Radio-iodination of VEGF, binding and cross-linking experiments.

The radio-iodination of $VEGF_{165}$ and $VEGF_{121}$ using IODO-BEADS was carried out as previously described (Soker et al., *J. Biol. Chem. 272*, 31582-31588 (1997)). The specific activity ranged from 40,000-100,000 cpm/ng protein. Binding and cross-linking experiments using ^{125}I-$VEGF_{165}$ and ^{125}I-$VEGF_{121}$ were performed as previously described (Gitay-Goren et al., *J. Biol. Chem. 267*, 6093-6098 (1992); Soker et al., *J. Biol. Chem. 271*, 5761-5767 (1996)). VEGF binding was quantitated by measuring the cell-associated radioactivity in a γ-counter (Beckman, Gamma 5500). The counts represent the average of three wells. All experiments were repeated at least

three times and similar results were obtained. The results of the binding experiments were analyzed by the method of Scatchard using the LIGAND program (Munson and Rodbard, 1980). ^{125}I-VEGF$_{165}$ and ^{125}I-VEGF$_{121}$ cross linked complexes were resolved by 6% SDS/PAGE and the gels were exposed to an X-Ray film. X-ray films were subsequently scanned by using an IS-1000 digital imaging system (Alpha Innotech Corporation)

Purification of $VEGF_{165}R$

Cross-linking of ^{125}I-$VEGF_{165}$ to cell surface receptors of 231 cells results in formation of a 165-175 kDa labeled complex (Soker et al., *J. Biol. Chem. 271*, 5761-5767 (1996)). These cells have about 1-2 x 10^5 $VEGF_{165}$ binding sites/cell. In contrast to $VEGF_{165}$, $VEGF_{121}$ does not bind to the 231 cells and does not form a ligand-receptor complex (Soker et al., *J. Biol. Chem. 271*, 5761-5767 (1996)). The relatively high $VEGF_{165}R$ number and the lack of any detectable KDR or Flt-1 mRNA in 231 cells (not shown) suggested that these cells would be a useful source for $VEGF_{165}R$ purification. Preliminary characterization indicated that $VEGF_{165}R$ is a glycoprotein and accordingly, a 231 cell lysate prepared from approximately 5 x 10^8 cells was absorbed onto a Con A Sepharose column. Bound proteins, eluted from the Con A column, were incubated with $VEGF_{165}$-Sepharose and the $VEGF_{165}$- affinity purified proteins were analyzed by SDS-PAGE and silver staining (Figure 9, lane 2). A prominent doublet with a molecular mass of about 130-135 kDa was detected. This size is consistent with the formation of a 165-175 kDa complex of 40-45 kDa $VEGF_{165}$ bound to receptors approximately 130-135 kDa in size (Figure 9, lane 1). The two bands were excised separately and N-terminal amino acid sequencing was carried out (Figure 1, right). Both the upper and lower bands had similar N-terminal amino acid sequences which showed high degrees of sequence homology to the predicted amino acid sequences in the N-terminal regions of mouse (Kawakami et al., *J. Neurobiol*, 29, 1-17 (1995)) and human neuroplilin-1 (NP-1) (He and Tessier-Lavigne, *Cell 90*739-751 (1997)).

Expression cloning of $VEGF_{165}R$ from 231 cell-derived mRNA

Concomitant with the purification, $VEGF_{165}R$ was cloned by expression cloning (Aruffo and Seed, *Proc. Natl. Acad. Sci.* USA 84, 8573-8577 (1987a); Aruffo and Seed, *EMBO J.*, 6, 3313-3316 (1987b); Gearing et al., *EMBO J.* 8,3667-3676 (1989)). For expression cloning, 231 cell mRNA was used to prepare a cDNA library of approximately 10^7 clones in a eukaryotic expression plasmid. *E. coli* transformed with the plasmid library were divided into pools. The DNA prepared from each pool were transfected into COS-7 cells in separate wells and individual cells were screened

for the ability to bind ^{125}I-VEGF$_{165}$ as detected by autoradiography of monolayers overlayed with photographic emulsion (Fig 2A). After three rounds of subpooling and screening, seven single positive cDNA clones were obtained. Figure 2B shows binding of ^{125}I-VEGF$_{165}$ to COS-7 cells transfected with one of these single positive clones (clone A2).

Restriction enzyme analysis revealed that six of the seven positive single clones had identical restriction digestion patterns but that one clone had a pattern that was different (not shown). Sequencing of one of these similar cDNA clones, clone A2 (Figure 3), showed it to be identical to a sequence derived from a human-expressed sequence tag data bank (dbEST). This sequence also showed a high percentage of homology to the sequence of mouse neuropilin, NP-1 (Kawakami et al., *J. Neurobiol 29*, 1-17 (1995)). After we had cloned human VEGF$_{165}$R, two groups reported the cloning of rat and human receptors for semaphorin III and identified them to be NP-1 (He and Tessier-Lavigne, *Cell 90*, 739-751 (1997); Kolodkin et al., *Cell 90*, 753-762 (1997)). The 231 cell-derived VEGF$_{165}$R cDNA sequence is virtually identical (see figure legend 3 for exceptions) to the human NP-1 sequence (He and Tessier-Lavigne, *Cell 90*, 739-751 (1997)). Significantly, the predicted amino acid sequence obtained by expression cloning (Figure 3) confirmed the identification of VEGF$_{165}$R as NP-1 that was determined by N-terminal sequencing (Figure 1), and we have therefore named this VEGF receptor, VEGF$_{165}$R/NP-1.

The human VEGF$_{165}$R/NP-1 cDNA sequence predicts an open reading frame (ORF) of 923 amino acids with two hydrophobic regions representing putative signal peptide and transmembrane domains (Figure 3). Overall, the sequence predicts ectodomain, transmembrane and cytoplasmic domains consistent with the structure of a cell surface receptor. The N-terminal sequence obtained via protein purification as shown in Figure 1 is downstream of a 21 amino acid putative hydrophobic signal peptide domain, thereby indicating directly where the signal peptide domain is cleaved and removed. The short cytoplasmic tail of 40 amino acids is consistent with results demonstrating that soluble VEGF$_{165}$R/NP-1 released by partial trypsin digestion of 231 cells is similar in size to intact VEGF$_{165}$R/NP-1 (not shown).

Sequence analysis of the one clone obtained by expression cloning that had a different restriction enzyme profile predicted an open reading frame of 931 amino acids with about a 47% homology to $VEGF_{165}R$/NP-1 (Figure 4). This human cDNA has a 93% sequence homology with rat neuropilin-2 (NP-2) and is identical to the recently cloned human NP-2 (Chen et al., *Neuron*, 19, 547-559 (1997)).

Expression of $VEGF_{165}R$/NP-1 in adult cell lines and tissues

Reports of NP-1 gene expression have been limited so far to the nervous system of the developing embryo (Takagi et al., *Dev. Biol. 122,* 90-100 (1987); Kawakami et al., *J. Neurobiol. 29,* 1-17 (1995); Takagi et al., *Dev. Biol.* 170, 207-222 (1995)). Cell surface $VEGF_{165}R$/NP-1 , however, is associated with non-neuronal adult cell types such as EC and a variety of tumor-derived cells (Soker et al., *J. Biol. Chem. 271,* 5761-5767 (1996)). Northern blot analysis was carried out to determine whether cells that crossed-linked ^{125}I-$VEGF_{165}$ also synthesized $VEGF_{165}R$/NP-1 mRNA. (Figure 5). $VEGF_{165}R$/NP-1 mRNA levels were highest in 231 and PC3 cells. $VEGF_{165}R$/NP-1 mRNA was detected to a lesser degree in HUVEC, LNCaP, EP-mel and RU-mel cells. There was little if any expression in MDA-MB-453 and WK-mel cells. The $VEGF_{165}R$/NP-1 gene expression patterns were consistent with our previous results showing that HUVEC, 231, PC3, LNCaP, EP-mel and RU-mel cells bind ^{125}I-$VEGF_{165}$ to cell surface $VEGF_{165}R$/NP-1 but that MDA-MB-453 and WK-mel cells do not (Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)).

$VEGF_{165}R$/NP-1 gene expression was analyzed also by Northern blot in a variety of adult tissues in comparison to KDR gene expression (Figure 6). $VEGF_{165}R$/NP-1 mRNA levels were relatively highly in adult heart and placenta and relatively moderate in lung, liver, skeletal muscle, kidney and pancreas. A relatively low level of $VEGF_{165}R$/NP-1 mRNA was detected in adult brain. Interestingly, previous analysis of NP-1 gene expression in mouse and chicken brain suggested that this gene was expressed primarily during embryonic development and was greatly diminished after birth (Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995); Takagi et al., *Dev. Biol. 170*, 207-222 (1995)). The tissue distribution of KDR mRNA was similar to that of $VEGF_{165}R$/NP-1 with the exception that it was not expressed highly in the

heart. These results indicate that $VEGF_{165}R$/NP-1 is expressed widely in adult non-neuronal tissue, including tissues in which angiogenesis occurs such as heart and placenta.

Characterization of $VEGF_{165}$ binding to $VEGF_{165}R$/NP-1

In order to characterize the binding properties of $VEGF_{165}R$/NP-1, porcine aortic endothelial (PAE) cells were transfected with the cDNA of $VEGF_{165}R$/NP-1. The PAE cells were chosen for these expression studies because they express neither KDR, Flt-1 (Waltenberger et al., *J. Biol. Chem. 269*, 26988-26995 (1994)) nor $VEGF_{165}R$. Stable cell lines synthesizing $VEGF_{165}R$/NP-1 (PAE/NP-1) were established and ^{125}I-$VEGF_{165}$ binding experiments were carried out (Fig 7). ^{125}I-$VEGF_{165}$ binding to PAE/NP-1 cells increased in a dose-dependent manner and reached saturation at approximately 30 ng/ml demonstrating that $VEGF_{165}R$/NP-1 is a specific $VEGF_{165}$ receptor (Figure 7A). Scatchard analysis of $VEGF_{165}$ binding revealed a single class of $VEGF_{165}$ binding sites with a K_d of approximately 3.2×10^{-10} M and approximately 3×10^{5} ^{125}I-$VEGF_{165}$ binding sites per cell (Figure 7B). Similar K_d values were obtained for several independently-generated PAE/NP-1 clones, although the receptor number varied from clone to clone (not shown). The K_d of 3×10^{-10} M for the PAE/NP-1 cell lines is consistent with the $2\text{-}2.8 \times 10^{-10}$ M K_d values obtained for $VEGF_{165}R$/NP-1 expressed naturally by HUVEC and 231 cells (Gitay-Goren et al., *J. Biol. Chem.* 267, 6093-6098 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). The binding of ^{125}I-$VEGF_{165}$ to PAE/NP-1 cells was enhanced by 1 μg/ml heparin (not shown), consistent with previous studies showing that heparin enhances ^{125}I-$VEGF_{165}$ binding to $VEGF_{165}R$/NP-1 on HUVEC and 231 cells (Gitay-Goren et al., *J. Biol. Chem.* 267, 6093-6098 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)).

Isoform-specific binding of VEGF to cells expressing $VEGF_{165}R$/NP-1

$VEGF_{165}$, but not $VEGF_{121}$, binds to $VEGF_{165}R$/NP-1 on HUVEC and 231 cells (Gitay-Goren et al., *J. Biol. Chem.* 271, 5519-5523 (1992); Soker et al., *J. Biol. Chem.* 271, 5761-5767 (1996)). To ascertain whether cells transfected with

$VEGF_{165}R$/NP-1 had the same binding specificity, PAE/NP-1 cells were incubated with ^{125}I-$VEGF_{165}$ or ^{125}I-$VEGF_{121}$ followed by cross-linking (Figure 8). ^{125}I-$VEGF_{165}$ did not bind to parental PAE cells (Figure 8, lane 3) but did bind to PAE/NP-1 cells via $VEGF_{165}R$/NP-1 (Figure 8, lane 4). The radiolabeled complexes formed with $VEGF_{165}R$/NP-1 were similar in size to those formed in HUVEC (Figure 8, lane 1) and PC3 cells (Figure 8, lane 2). On the other hand, ^{125}I-$VEGF_{121}$, did not bind to either parental PAE (Figure 8, lane 7) or to PAE/NP-1 cells (Figure 8, lane 8). These results demonstrate that the VEGF isoform-specific binding that occurs with cells expressing endogenous $VEGF_{165}R$/NP-1 such as HUVEC, 231 and PC3 cells, can be replicated in cells transfected with $VEGF_{165}R$/NP-1 cDNA and support the finding that $VEGF_{165}R$ and NP-1 are identical.

Co-expression of $VEGF_{165}R$/NP-1 and KDR
modulates $VEGF_{165}$ binding to KDR

To determine whether expression of $VEGF_{165}R$/NP-1 had any effect on $VEGF_{165}$ interactions with KDR, PAE cells that were previously transfected with KDR cDNA to produce stable clones of PAE/KDR cells (Waltenberger et al., *J. Biol. Chem.* 269, 26988-26995 (1994)), were transfected with $VEGF_{165}R$/NP-1 cDNA and stable clones expressing both receptors (PAE/KDR/NP-1) were obtained. These cells bound ^{125}I-$VEGF_{165}$ to KDR (Figure 8, lane 6, upper complex) and to $VEGF_{165}R$/NP-1 (Figure 8, lane 6, lower complex) to yield a cross-linking profile similar to HUVEC (Figure 8, lane 1). On the other hand, the PAE/KDR/NP-1 cells bound ^{125}I-$VEGF_{121}$ to form a complex only with KDR (Figure 8, lanes 9 and 10), consistent with the inability of $VEGF_{121}$ to bind $VEGF_{165}R$/NP-1.

It appeared that in cells co-expressing KDR and $VEGF_{165}R$/NP-1 (Figure 8, lane 6), the degree of ^{125}I-$VEGF_{165}$-KDR 240 kDa complex formation was enhanced compared to the parental PAE/KDR cells (Figure 8, lane 5). These results were reproducible and the degree of ^{125}I-$VEGF_{165}$-KDR 240 kDa complex formation in different clones was correlated positively with the levels of $VEGF_{165}R$/NP-1 expressed (not shown). However, it could not be ruled out definitively that these differential KDR binding results were possibly due to clonal selection post-transfection.

Therefore, parental PAE/KDR cells were transfected with $VEGF_{165}R$/NP-1 cDNA and ^{125}I-$VEGF_{165}$ was bound and cross-linked to the cells three days later in order to avoid any diversity of KDR expression among individual clones (Figure 9). A labeled 240 kDa complex containing KDR was formed in parental PAE/KDR cells (Fig 9, lane 1) and in PAE/KDR cells transfected with the expression vector (Figure 9, lane 2). However, when ^{125}I-$VEGF_{165}$ was cross-linked to PAE/KDR cells transiently expressing $VEGF_{165}R$/NP-1, a more intensely labeled 240 kDa complex, about 4 times greater, was observed (Figure 9, lane 3), compared to parental PAE/KDR cells (Figure 9, lane1) and PAE/KDR cells transfected with expression vector (Figure 9, lane 2). These results suggest that co-expression of KDR and $VEGF_{165}R$/NP-1 genes in the same cell enhances the ability of $VEGF_{165}$ to bind to KDR.

A GST-VEGF Exon 7+8 fusion protein inhibits $VEGF_{165}$ binding to $VEGF_{165}R$/NP-1 and KDR

We have shown that ^{125}I-$VEGF_{165}$ binds to $VEGF_{165}R$/NP-1 through its exon 7-encoded domain (Soker et al., *J. Biol. Chem. 271,* 5761-5767 (1996)). In addition, a GST fusion protein containing the peptide encoded by VEGF exon 7+8 (GST-Ex 7+8), inhibits completely the binding of ^{125}I-$VEGF_{165}$ to $VEGF_{165}R$/NP-1 associated with 231 cells and HUVEC (Soker et al., *J. Biol. Chem. 271,* 5761-5767 (1996); Soker et al., *J. Biol. Chem. 272*, 31582-31588 (1997)). When, added to PAE/NP-1 cells, the fusion protein completely inhibited binding to $VEGF_{165}R$/NP-1 (Figure 10, lane 2 compared to lane 1). On the other hand, it did not inhibit ^{125}I-$VEGF_{165}$ binding at all to KDR (Figure 10, lane 4 compared to lane 3). Thus, these results demonstrate that GST-Ex 7+8 binds directly to $VEGF_{165}R$/NP-1 but does not bind to KDR. The effects of GST-Ex 7+8 are different, however, in cells co-expressing both $VEGF_{165}R$/NP-1 and KDR (PAE/KDR/NP-1). Consistent with the results in Figures 8 and 9, the degree of ^{125}I-$VEGF_{165}$ binding to KDR in PAE/KDR/NP-1 cells (Figure 10, lane 5) was greater than to the parental PAE/KDR cells (Figure 10, lane 3). Interestingly, in PAE/KDR/NP-1 cells, GST-Ex 7+8 inhibited not only ^{125}I-$VEGF_{165}$ binding to $VEGF_{165}R$/NP-1 completely as expected, but it also inhibited binding to KDR substantially which was unexpected (Figure 10, lane 6 compared to lane 5). In the

presence of GST-Ex 7+8, binding of ^{125}I-$VEGF_{165}$ to KDR in these cells was reduced to the levels seen in parental PAE/KDR cells not expressing $VEGF_{165}$R/NP-1 (Figure 10, lane 6 compared to lanes 3 and 4). Since the fusion protein does not bind directly to KDR, these results suggest that inhibiting the binding of ^{125}I-$VEGF_{165}$ to $VEGF_{165}$R/NP-1 directly, inhibits its binding to KDR indirectly. Taken together, the results in Figures 8, 9 and 10 suggest that interactions of $VEGF_{165}$ with $VEGF_{165}$R/NP-1 enhance VEGF interactions with KDR.

Neuropilin-1 is an isoform-specific VEGF $_{165}$ receptor

Recently, we described a novel 130-135 kDa VEGF cell surface receptor that binds $VEGF_{165}$ but not $VEGF_{121}$ and that we named, accordingly, $VEGF_{165}$R (Soker et al., *J. Biol. Chem. 271,* 5761-5767 (1996)). We have now purified $VEGF_{165}$R, expression cloned its cDNA, and shown it to be identical to human neuropilin-1 (NP-1) (He and Tessier-Lavigne, *Cell 90* 739-751 (1997)). The evidence that $VEGF_{165}$R is identical to NP-1 and that NP-1 serves as a receptor for $VEGF_{165}$ is as follows: i) purification of $VEGF_{165}$R protein from human MDA-MB-231 (231) cells using VEGF affinity, yielded a 130-140 kDa doublet upon SDS-PAGE and silver stain. N-terminal sequencing of both proteins yielded the same N-terminal sequence of 18 amino acids that demonstrated a high degree of homology to mouse NP-1 (Kawakami et al., *J. Neurobiol. 29*, 1-17 (1995)); ii) After we purified $VEGF_{165}$R from human 231 cells, the cloning of human NP-1 was reported (He and Tessier-Lavigne, *Cell 90*, 739-751 (1997)) and the N-terminal sequence of human $VEGF_{165}$R was found to be identical to a sequence in the N-terminal region of human NP-1; iii) Expression cloning using a 231 cell cDNA library resulted in isolation of several cDNA clones and their sequences were identical to the human NP-1 cDNA sequence (He and Tessier-Lavigne, *Cell 90*, 739-751 (1997)). The combination of purification and expression cloning has the advantage over previous studies where only expression cloning was used (He and Tessier-Lavigne, *Cell 90*, 739-751 (1997); Kolodkin et al., *Cell 90*, 753-762 (1997)), in allowing unambiguous identification of the NP-1 protein N-terminus; iv) Northern blot analysis of NP-1 gene expression was consistent with previous ^{125}I-$VEGF_{165}$ cross-linking experiments (Soker et al., *J. Biol. Chem. 271,* 5761-5767

(1996)). Cells that bound $VEGF_{165}$ to $VEGF_{165}R$ synthesized relatively abundant NP-1 mRNA while cells that showed very little if any $VEGF_{165}$ binding, did not synthesize much if any NP-1 mRNA; v) when NP-1 was expressed in PAE cells, the transfected , but not the parental cells, were able to bind $VEGF_{165}$ but not $VEGF_{121}$, consistent with the isoform specificity of binding previously shown for HUVEC and 231 cells (Soker et al., *J. Biol. Chem. 271,* 5761-5767 (1996)). Furthermore, the K_d of ^{125}I -$VEGF_{165}$ binding of to PAE expressing NP-1 was about 3 x 10^{-10} M, consistent with previous K_d binding values of 2-2.8 x 10^{-10} M for 231 cells and HUVEC (Soker et al., *J. Biol. Chem. 271,* 5761-5767 (1996)); and vi) The binding of $VEGF_{165}$ to cells expressing NP-1 post-transfection was more efficient in the presence of heparin as was the binding of this ligand to HUVEC and 231 cells (Gitay-Goren et al., *J. Biol. Chem. 267,* 6093-6098 (1992); Soker et al., *J. Biol. Chem. 271,* 5761-5767 (1996)). Taken together, these results show not only that $VEGF_{165}R$ is identical to NP-1 but that it is a functional receptor that binds $VEGF_{165}$ in an isoform-specific manner. Accordingly, we have named this VEGF receptor $VEGF_{165}R$/NP-1.

In addition to the expression cloning of $VEGF_{165}R$/NP-1 cDNA, another human cDNA clone was isolated whose predicted amino acid sequence was 47% homologous to that of $VEGF_{165}R$/NP-1 and over 90% homologous to rat neuropilin-2 (NP-2) which was recently cloned (Kolodkin et al., *Cell 90*, 753-762 (1997)). NP-2 binds members of the collapsin/semaphorin family selectively (Chen et al., *Neuron 19*, 547-559 (1997)).

The discovery that NP-1 serves as a receptor for $VEGF_{165}$ was a surprise since NP-1 had previously been shown to be associated solely with the nervous system during embryonic development (Kawakami et al., *J. Neurobiol.* 29, 1-17 (1995); Takagi et al., *Dev. Biol.* 170, 207-222 (1995)) and more recently as a receptor for members of the collapsin/semaphorin family (He and Tessier-Lavigne, *Cell 90*739-751 (1997); Kolodkin et al., *Cell 90*, 753-762 (1997)). NP-1 is a 130-140 kDa transmembrane glycoprotein first identified in the developing Xenopus optic system (Takagi et al., *Dev. Biol.* 122, 90-100 (1987); Takagi et al., *Neuron 7,* 295-307 (1991)). NP-1 expression in the nervous system is highly regulated spatially and temporally during development and in particular is associated with those

developmental stages when axons are actively growing to form neuronal connections.(Fujisawa et al., *Dev. Neurosci. 17,* 343-349 (1995); Kawakami et al., *J. Neurobiol* 29, 1-17 (1995); Takagi et al., *Dev. Biol.* 170, 207-222 (1995)). The NP-1 protein is associated with neuronal axons but not the stomata (Kawakami et al., *J. Neurobiol* 29, 1-17 (1995)). Functionally, neuropilin has been shown to promote neurite outgrowth of optic nerve fibers in vitro (Hirata et al., *Neurosci. Res. 17*, 159-169 (1993)) and to promote cell adhesiveness (Tagaki et al., *Dev. Biol. 170*, 207-222 (1995)). Targeted disruption of NP-1 results in severe abnormalities in the trajectory of efferent fibers of the peripheral nervous system (Kitsukawa et al., *Neuron 19,* 995-1005 (1997)). Based on the these studies, it has been suggested that NP-1 is a neuronal cell recognition molecule that plays a role in axon growth and guidance (Kawakami et al., *J. Neurobiol. 29*, 1-17 (1995); He and Tessier-Lavigne, *Cell 90,* 739-751 (1997); Kitsukawa et al., *Neuron 19*, 995-1005 1997; Kolodkin et al., *Cell 90*, 753-762 (1997)).

Our results are the first to show that $VEGF_{165}R$/NP-1 is also expressed in adult tissues, in contrast to the earlier studies that have shown that NP-1 expression in Xenopus, chicken and mouse is limited to the developmental and early post-natal stages (Fujisawa et al., *Dev. Neurosci. 17*, 343-349 (1995); Kawakami et al., *J. Neurobiol. 29*, 1-17 (1995); Takagi et al., *Dev. Biol.*170, 207-222 (1995)). For example, in mice, NP-1 is expressed in the developing nervous system starting in the dorsal root ganglia at day 9 and ceases at day 15 (Kawakami et al., *J. Neurobiol. 29*, 1-17 (1995). Our Northern blot analysis of human adult tissue demonstrates relatively high levels of $VEGF_{165}R$/NP-1 mRNA transcripts in heart, placenta, lung, liver, skeletal muscle, kidney and pancreas. Interestingly, there is very little relative expression in adult brain, consistent with the mouse nervous system expression studies (Kawakami et al., *J. Neurobiol. 29*, 1-17 (1995)). $VEGF_{165}R$/NP-1 is also expressed in a number of cultured non-neuronal cell lines including EC and a variety of tumor-derived cells. A possible function of $VEGF_{165}R$/NP-1 in these cells is to mediate angiogenesis as will be discussed below.

In addition, NP-1 has been identified as a receptor for the collapsin/semaphorin family by expression cloning of a cDNA library obtained from

rat E14 spinal cord and dorsal root ganglion (DRG) tissue (He and Tessier-Lavigne, *Cell 90*, 739-751 (1997); Kolodkin et al., *Cell 90*, 753-762 (1997)). The collapsin/semaphorins (collapsin-D-1/Sema III/Sem D) comprise a large family of transmembrane and secreted glycoproteins that function in repulsive growth cone and axon guidance (Kolodkin et al., *Cell 75,* 1389-1399 (1993)). The repulsive effect of sema III for DRG cells was blocked by anti-NP-1 antibodies (He and Tessier-Lavigne, *Cell 90*, 739-751 (1997); Kolodkin et al., *Cell 90*, 753-762 (1997)). The K_d of sema III binding to NP-1, 0.15- 3.25 x 10^{-10} M (He and Tessier-Lavigne, *Cell 90*, 739-751 (1997); Kolodkin et al., *Cell 90*, 753-762 (1997)) is similar to that of $VEGF_{165}$ binding $VEGF_{165}$/NP-1, which is about 3 x 10^{-10} M. These results indicate that two structurally different ligands with markedly different biological activities, VEGF-induced stimulation of EC migration and proliferation on one hand, and sema III-induced chemorepulsion of neuronal cells, on the other hand, bind to the same receptor and with similar affinity. An interesting question is whether the two ligands bind to the same site on $VEGF_{165}$R/NP-1 or to different sites. $VEGF_{165}$R/NP-1 has five discrete domains in its ectodomain, and it has been suggested that this diversity of protein modules in NP-1 is consistent with the possibility of multiple binding ligands for NP-1 (Takagi et al., *Neuron 7*, 295-307 (1991); Feiner et al., *Neuron 19* 539-545 (1997); He and Tessier-Lavigne, *Cell 90* 739-751 (1997). Preliminary analysis does not indicate any large degree of sequence homology between sema III and VEGF exon 7 which is responsible for VEGF binding to $VEGF_{165}$R/NP-1 (Soker et al., *J. Biol. Chem. 271*, 5761-5767 (1996)). However there may be some 3-dimensional structural similarities between the two ligands. Since both neurons and blood vessels display branching and directional migration , the question also arises as to whether $VEGF_{165}$ displays any neuronal guidance activity and whether sema III has any EC growth factor activity. These possibilities have not been examined yet. However, it may be that VEGF requires two receptors, KDR and NP-1 for optimal EC growth factor activity (Soker et al., *J. Biol. Chem. 272*, 31582-31588 (1997)) and that sema III requires NP-1 and an as yet undetermined high affinity receptor for optimal chemorepulsive activity (Feiner et al., *Neuron 19*, 539-545 (1997;) He and Tessier-Lavigne, *Cell 90,* 739-751 (1997); Kitsukawa et al., *Neuron 19*, 995-1005 (1997)), so

that the presence of NP-1 alone might not be sufficient for these ligands to display novel biological activities. Future studies will determine whether there are any connections between the mechanisms that regulate neurogenesis and angiogenesis.

$VEGF_{165}R$/NP-1 role angiogenesis

$VEGF_{165}R$/NP-1 modulates the binding of $VEGF_{165}$ to KDR, a high affinity RTK that is an important regulator of angiogenesis as evidenced by KDR knock out experiments in mice (Shalaby et al., *Nature 376*, 62-66 (1995). The affinity of KDR for $VEGF_{165}$ is about 50 times greater than for $VEGF_{165}R$/NP-1 (Gitay-Goren et al., *J. Biol. Chem. 287*, 6003-6096 (1992); Waltenberger et al., *J. Biol. Chem. 269*, 26988-26995 (1994)). When $VEGF_{165}R$/NP-1 and KDR are co-expressed, the binding of ^{125}I-$VEGF_{165}$ to KDR is enhanced by about 4-fold compared to cells expressing KDR alone. The enhanced binding can be demonstrated in stable clones co-expressing $VEGF_{165}R$/NP-1 and KDR (PAE/KDR/NP-1 cells), and also in PAE/KDR cells transfected transiently with $VEGF_{165}R$/NP-1 cDNA where clonal selection does not take place. Conversely, when the binding of ^{125}I-$VEGF_{165}$ to $VEGF_{165}R$/NP-1 in PAE/KDR/NP-1 cells is inhibited completely by a GST fusion protein containing VEGF exons 7+8 (GST-Ex 7+8), the binding to KDR is inhibited substantially, down to the levels observed in cells expressing KDR alone. The fusion protein binds to $VEGF_{165}R$/NP-1 directly but is incapable of binding to KDR directly (Soker et al., *J. Biol. Chem. 272*, 31582-31588 (1997)). Although, not wishing to be bound bytheory, we believe that $VEGF_{165}$ binds to $VEGF_{165}R$/NP-1 via the exon 7-encoded domain and facilitates $VEGF_{165}$ binding to KDR via the exon 4-encoded domain (Figure 11). $VEGF_{165}R$/NP-1, with its relatively high receptor/cell number, about 0.2-2 x 10^5 (Gitay-Goren et al., *J. Biol. Chem. 287*, 6003-6096 (1992); Soker et al., *J. Biol. Chem. 271*, 5761-5767 (1996)), appears to serve to concentrate $VEGF_{165}$ on the cell surface, thereby providing greater access of $VEGF_{165}$ to KDR. Alternatively, binding to $VEGF_{165}R$/NP-1, $VEGF_{165}$ undergoes a conformational change that enhances its binding to KDR. The end result would be elevated KDR signaling and increased VEGF activity. Although we can demonstrate enhanced binding to KDR, to date we have not been able to demonstrate enhanced VEGF mitogenicity for PAE/KDR/NP-1

cells compared to PAE/KDR cells. One reason is that these cell lines do not proliferate readily in response to VEGF as do HUVEC (Waltenberger et al., *J. Biol. Chem. 269*, 26988-26995 (1994). Nevertheless, we have shown that $VEGF_{165}$, which binds to both KDR and $VEGF_{165}R$/NP-1, is a better mitogen for HUVEC than is $VEGF_{121}$, which binds only to KDR (Keyt et al., *J. Biol. Chem. 271*, 5638-5646 (1996b); Soker et al., *J. Biol. Chem. 272*, 31582-31588 (1997). Furthermore, inhibiting $VEGF_{165}$ binding to $VEGF_{165}R$/NP-1 on HUVEC by GST-EX 7+8, inhibits binding to KDR and also inhibits $VEGF_{165}$-induced HUVEC proliferation, down to the level induced by $VEGF_{121}$ (Soker et al., *J. Biol. Chem. 272*, 31582-31588 (1997)). Taken together, these results suggest a role for $VEGF_{165}R$/NP-1 in mediating $VEGF_{165}$, but not $VEGF_{121}$ mitogenic activity. The concept that dual receptors regulate growth factor binding and activity has been previously demonstrated for TGF-β, bFGF and NGF (Lopez-Casillas et al., *Cell 67*, 785-795 (1991); Yayon et al., *Cell 64*, 841-848 (1991; Barbacid, *Curr. Opin. Cell Biol.* 7, 148-155 (1995)).

Another connection between $VEGF_{165}R$/NP-1 and angiogenesis comes from studies in which NP-1 was overexpressed ectopically in transgenic mice (Kitsuskawa et al., *Develop.* 121, 4309-4318 (1995)). NP-1 overexpression resulted in embryonic lethality and the mice died *in utero* no later than on embryonic day 15.5 and those that survived the best had lower levels of NP-1 expression. Mice overexpressing NP-1 displayed morphologic abnormalities in a limited number of non-neural tissues such as blood vessels, the heart and the limbs. NP-1 was expressed in both the EC and in the mesenchymal cells surrounding the EC. The embryos possessed excess and abnormal capillaries and blood vessels compared to normal counterparts and in some cases dilated blood vessels as well. Some of the chimeric mice showed hemorrhaging, mainly in the head and neck. These results are consistent with the possibility that ectopic overexpression of $VEGF_{165}R$/NP-1 results in inappropriate $VEGF_{165}$ activity, thereby mediating enhanced and/or aberrant angiogenesis. Another piece of evidence for a link between NP-1 and angiogenesis comes from a recent report showing that in mice targeted for disruption of the NP-1 gene, the embryos have severe abnormalities in the peripheral nervous system but that their death *in utero* at days 10.5-12.5 is most

probably due to anomalies in the cardiovascular system (Kitsukawa et al., *Neuron 19*, 995-1005 (1997)).

VEGF$_{165}$R/NP –1 is associated with tumor-derived cells

The greatest degree of VEGF$_{165}$R/NP-1 expression that we have detected so far occurs in tumor-derived cells such as 231 breast carcinoma cells and PC3 prostate carcinoma cells, far more than occurs in HUVEC. The tumor cells express abundant levels of VEGF$_{165}$R/NP-1 mRNA and about 200,000 VEGF$_{165}$ receptors/cell (Soker et al., *J. Biol. Chem. 271*, 5761-5767 (1996)). On the other hand, these tumor cells do not express KDR or Flt-1 so that VEGF$_{165}$R/NP-1 is the only VEGF receptor associated with these cells. The tumor cells are therefore useful for testing whether VEGF$_{165}$R/NP-1 is a functional receptor for VEGF$_{165}$ in the absence of a KDR background. To date, we have not been able to show that VEGF$_{165}$R/NP-1 mediates a VEGF$_{165}$ signal in tumor-derived cells as measured by receptor tyrosine phopshorylation. Nevertheless, VEGF$_{165}$ might have an effect on tumor cells by inducing some, as yet undetermined activity such as enhanced survival, differentiation, or motility. A recent report has demonstrated that glioma cells express a 190 kDa protein that binds VEGF$_{165}$ but not VEGF$_{121}$ efficiently (Omura et al., *J. Biol. Chem. 272*, 23317-23322 (1997)). No stimulation of tyrosine phosphorylation could be demonstrated upon binding of VEGF$_{165}$ to this receptor. Whether the 190 kDa isoform-specific receptor is related to VEGF$_{165}$R/NP-1 is not known presently.

VEGF$_{165}$R/NP-1 may have a storage and sequestration function for VEGF$_{165}$. One might envision that VEGF$_{165}$ is produced by a tumor cell and binds to VEGF$_{165}$R/NP-1 on that cell via the exon 7-encoded domain (Soker et al., *J. Biol. Chem. 271*, 5761-5767 (1996)). The stored VEGF$_{165}$ could be then released to stimulate tumor angiogenesis in a paracrine manner. Alternatively, VEGF$_{165}$R/NP-1 may mediate a juxtacrine effect in which VEGF$_{165}$ is bound to VEGF$_{165}$R/NP-1 on a tumor cell via the exon 7-encoded domain and is also bound to KDR on a neighboring EC via the exon 4-encoded domain (Keyt et al., *J. Biol. Chem. 271*, 5638-5646 (1996b)). Such a mechanism could result in a more efficient way for tumor cells to attract EC, thereby enhancing tumor angiogenesis.

In summary, we have demonstrated by independent purification and expression cloning methods that the VEGF isoform specific receptor, $VEGF_{165}R$, is identical to NP-1, a cell surface protein previously identified as playing a role in embryonic development of the nervous system and as being a receptor for the collapsins/semaphorins. Furthermore, binding to $VEGF_{165}R$/NP-1 enhances the binding of $VEGF_{165}$ to KDR on EC and tumor cells.

Experimental Rationale

We have discovered that tumor cell neuropilin-1 mediates tumor cell motility and thereby metastasis. In a Boyden chamber motility assay, $VEGF_{165}$ (50 ng/ml) stimulates 231 breast carcinoma cell motility in a dose-response manner, with a maximal 2-fold stimulation (Fig. 15A). On the other hand, $VEGF_{121}$ has no effect on motility of these cells (Fig. 15B). Since 231 cells do not express KDR or Flt-1, these results suggest that tumor cells are directly responsive to $VEGF_{165}$ and that $VEGF_{165}$ might signal tumor cells via neuropilin-1. Possible candidates for mediating $VEGF_{165}$-induced motility of carcinoma cells are PI3-kinase (PI3-K) (Carpenter, et al. (1996) *Curr. Opin. Cell Biol.* 8: 153-158.). Since 231 cells do not express KDR or Flt-1, these results suggest that tumor cells are directly responsive to $VEGF_{165}$ and that $VEGF_{165}$ might signal tumor cells via neuropilin-1.

The other type of evidence is that neuropilin-1 expression might be associated with tumor cell motility. We have analyzed two variants of Dunning rat prostate carcinoma cells, AT2.1 cells, which are of low motility and low metastatic potential, and AT3.1 cells, which are highly motile, and metastatic. Cross-linking and Northern blot analysis show that AT3.1 cells express abundant neuropilin-1, capable of binding $VEGF_{165}$, while AT2.1 cells don't express neuropilin-1 (Fig. 16). Immunostaining of tumor sections confirms the expression of neuropilin-1 in AT3.1, but not AT2.1 tumors (Fig. 17). Furthermore, the immunostaining shows that in subcutaneous AT3.1 and PC3 tumors, the tumor cells expressing neuropilin-1 are found preferentially at the invading front of the tumor/dermis boundary (Fig. 17). To determine more directly whether neuropilin-1 expression is correlated with enhanced motility, neuropilin-1 was overexpressed in AT2.1 cells (Fig. 18). Three stable clones of AT2.1 cells overexpressing neuropilin-1 had enhanced motility in the Boyden chamber assay.

These results indicate that expression of neuropilin-1 in AT2.1 cells enhances their motility. Taken together, it appears that neuropilin-1 expression on tumor cells is associated with the motile, metastatic phenotype.

EXAMPLE 2

Experimental Procedures

1. Collapsin/semaphorins. Expression plasmids for expressing and purifying His-tagged collapsin-1 from transfected 293T cells can be produced according to the methods of (Koppel, et al. (1998) J. Biol. Chem. 273: 15708-15713, Feiner, et al. (1997) *Neuron* 19: 539-545.). Expression vectors for expressing sema E and sema IV alkaline phosphate (AP) conjugates in cells are disclosed in (He Z, Tessier-Lavigne M. (1997). Neuropilin is a receptor for the axonal chemorepellent semaphorin III. Cell 90: 739-751.). Migration was measured in a Boyden chamber Falk, et al., *J. Immunol.* 118:239-247 (1980) with increasing concentration of recombinant chick collapsin-1 in the bottom well and PAE cell transfectants in the upper well.

2. Aortic Ring Assay. 200 gram rats were sacrificed and the aorta is dissected between the aortic arch and kidney artery and the adipofibrotic tissue around the aorta was removed. Aortic rings were sliced at 1 mm intervals and embedded in type I collagen gels. Each ring was cultured in one well of a 48-well plate with serum-free endothelial cell medium (GIBCO). The number of microvessels were counted in each ring using a phase microscope (Miao, et al. (1997). *J. Clin. Invest.* 99: 1565-1575.).

We established several endothelial cell lines by transfection of parental porcine aortic endothelial cells (PAE), which normally do not express VEGF receptors (Soker, et al.(1998)*Cell* 92: 735-745). The cell lines included PAE cells expressing neuropilin-1 alone (PAE/NP1), PAE cells expressing KDR alone (PAE/KDR) and PAE cells expressing both neuropilin-1 and KDR (PAE/NP1/KDR). Collapsin-1 was obtained from Dr. Jon Raper, University of Pennsylvania (Luo,et al.(1993)*Cell* 75: 217-227.).

Binding studies demonstrated that ^{125}I-collapsin-1 could bind to PAE/NP1 cells and PAE/NP1/KDR cells but not at all to PAE or PAE/KDR cells.

In a Boyden chamber assay, collapsin-1 at 50-100 collapsin units/ml (CU) inhibited the basal migration of PAE/NP and PAE/NP1/KDR cells by 70% but had no inhibitory effect, whatsoever, on basal PAE or PAE/KDR cell migration (Fig. 20). This effect is fairly potent since 1 CU represents about 3 ng/ml protein. The collapsin-1 inhibitory effect was inhibited by anti-neuropilin-1 antibodies. These results indicate that collapsins can inhibit the migration of non-neuronal endothelial cells as long as they express neuropilin-1.

Collapsin-1 inhibited the migration of PAE/NP and PAE/NP/KDR cells in the presence of $VEGF_{165}$, to the same degree, the baseline being higher. We have also found that addition of collapsin in a rat aortic ring assay (a model for angiogenesis *in vitro*) inhibits the migration of endothelial cells out of the ring, and endothelial tube formation (Fig. 21).

The references cited throughout the specification are incorporated herein by reference.

The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in the art without departing from the spirit and the scope of the appended claims.

SEQUENCE LISTING

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What is claimed:

1. A neuropilin antagonist that binds neuropilin and has VEGF antagonist activity as determined by the human umbilical vein endothelial cell (HUVEC) proliferation assay using $VEGF_{165}$.

2. The neuropilin antagonist of claim 1, wherein the antagonist is an antibody that specifically inhibits binding of VEGF to a neuropilin receptor.

3. The neuropilin antagonist of claim 1, wherein the antagonist is a member of the semaphorin/collapsin family or a fragment thereof.

4. The neuropilin antagonist of claim 1, wherein the member of the semaphorin/collapsin family is collapsin-1.

5. An antibody directed against a neuropilin receptor, wherein said antibody specifically inhibits binding of VEGF to the receptor.

6. The antibody of claim 5, wherein the neuropilin is NP-1 or NP-2.

7. A method for identifying an antagonist which binds to a neuropilin, comprising exposing the neuropilin to the molecule suspected of binding thereto and determining binding of the molecule to the receptor.

8. The method of claim 7, wherein the neuropilin is NP-1 or NP-2.

9. A method of inhibiting metastasis in a patient having malignant cells which comprises:

(a) determining whether the patient's malignant cells express a neuropilin, and if they do adding a compound that interferes with the neuropilin.

10. The method of claim 9, wherein the compound interferes with the binding activity of the neuropilin.

11. The method of claim 10, wherein the compound is a antibody that specifically binds neuropilin or a neuropilin antagonist.

12. The method of claim 9, wherein the compound interferes with neuropilin expression.

13. The method of claim 11, wherein the compound is a member of the semaphorin/collapsin family or a fragment thereof.

14.. The method of claim 12, wherein the member of the semaphorin/collapsin family is collapsin-1.

15. The method of claim 9, wherein the malignant cell is a breast or prostate cell or a melanoma.

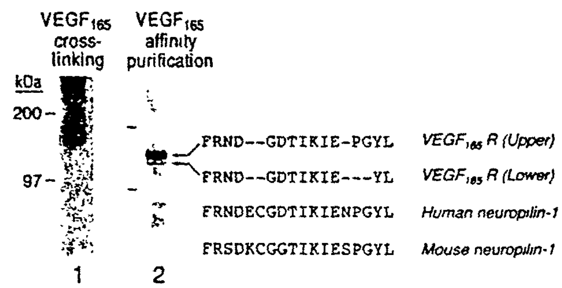
16. The method of claim 9, wherein the neuropilin is $VEGF_{165}R/NP\text{-}1$ or NP-2.

~~17~~. Use of a member of the semaphorin/collapsin family in the preparation of a medicament for the treatment of a disease or disorder associated with VEGF.

ABSTRACT

The present invention relates to antagonists of neuropilin receptor fuction and use thereof in the treatment of cancer, particularly metastatic cancer, and angiogenic diseases.

Figure 1



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Human neuropilin-1
Mouse neuropilin-1
1
2

Figure 2A

Figure 2B

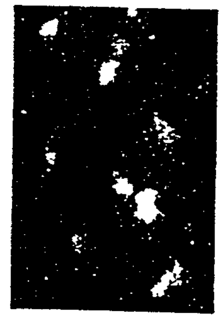

Figure 3

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Figure 4

Comparative Deduced Amino Acid Sequences of Human $VEGF_{165}R/NP$ and $VEGF_{165}R/NP$-1

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VEGF165R/NP-1      MERGLPLLCAV-LAL------VLA-PA---GAFR-NDKCGDTIKIESPGY

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NP-1               LTSPGYPHSYHPSEK-CEWLIQ-APDPYQRIMINFNPHFDLEDRDCKYDY

NP-2           101 IEIRDGDSESADLLGKHCGNIAPPTIISSGSMLYIKFTSDYARQGAGFSL  150
NP-1               VEVFDGENENGHFRGKFCGKIAPPPVVSSGPFLFIKFVSDYETHGAGFSI

NP-2           151 RYEIFKTGSEDCSKNFTSPNGTIESPGFPEKYPHN-LDCTFTIL-AKPKM  200
NP-1               RYEIFKRGPE-CSQNYTTPSGVIKSPGFPEKYP-NSLECTY-IVFA-PKM

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Figure 5

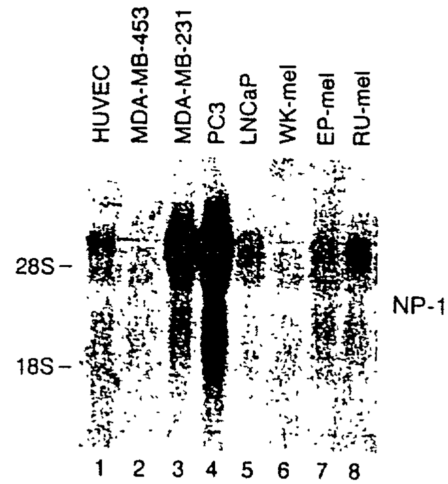

Figure 6

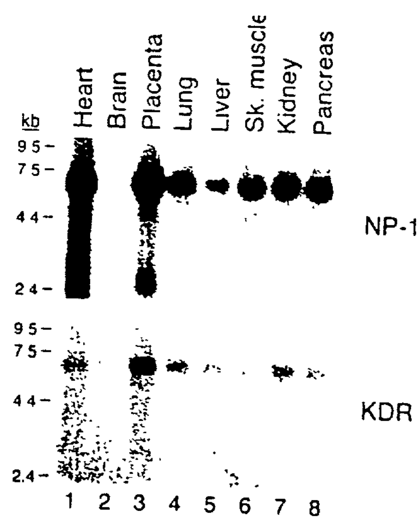

Figure 7A

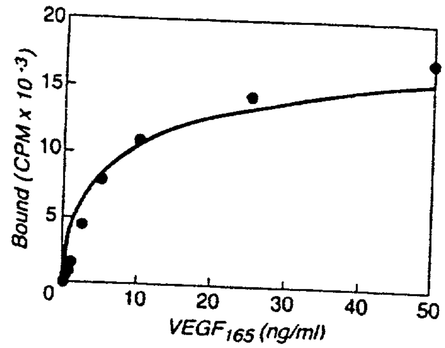

Figure 7B

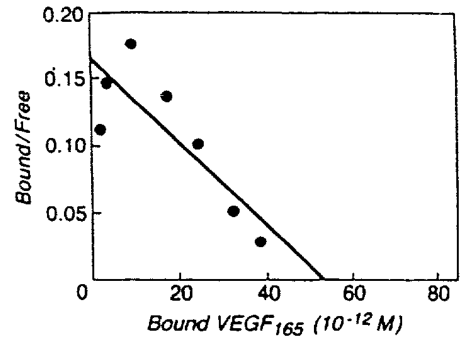

Figure 8

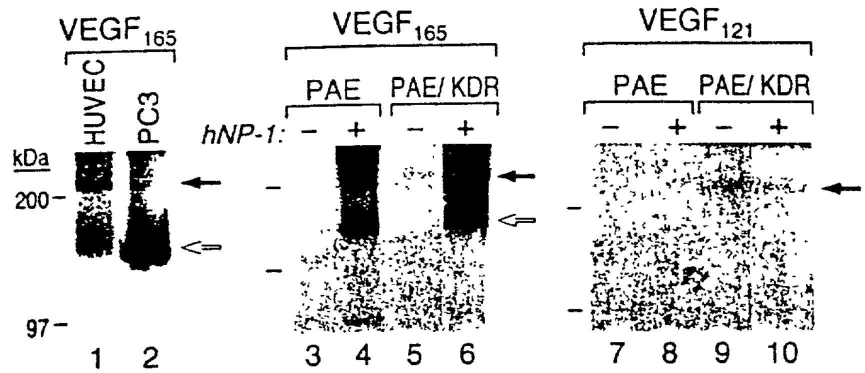

Figure 9

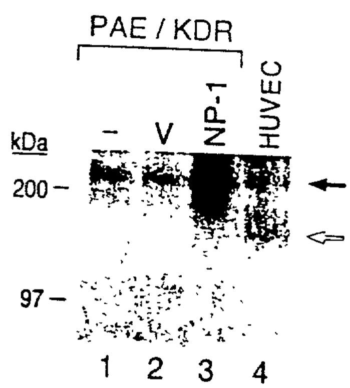

Figure 10

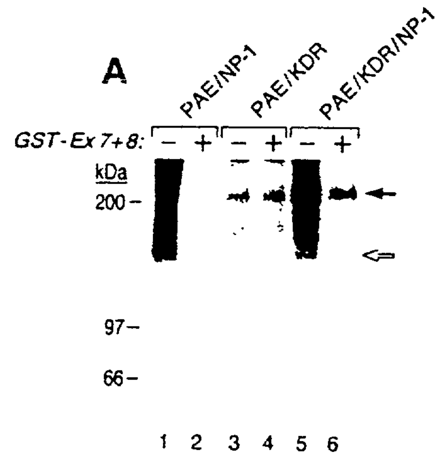

Figure 11A

KDR alone

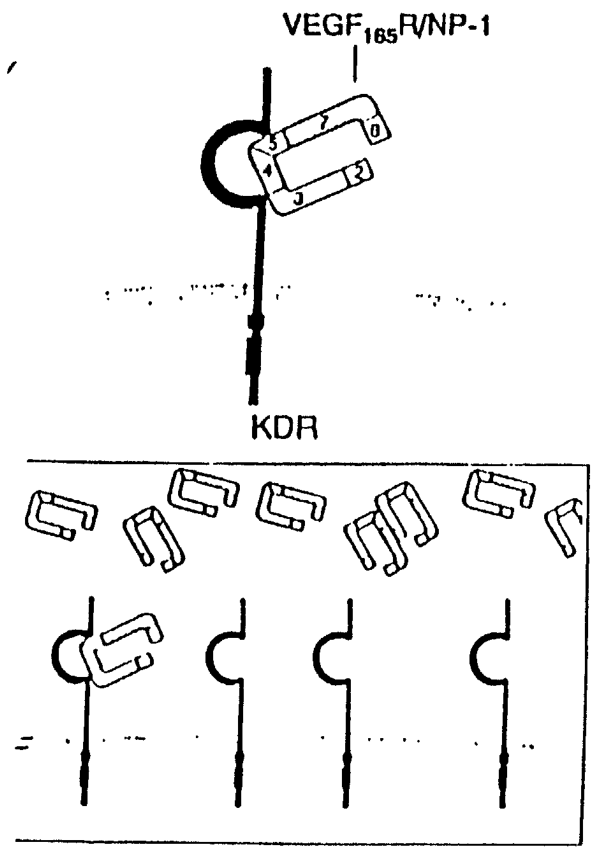

Figure 11B

KDR + $VEGF_{165}R/NP-1$

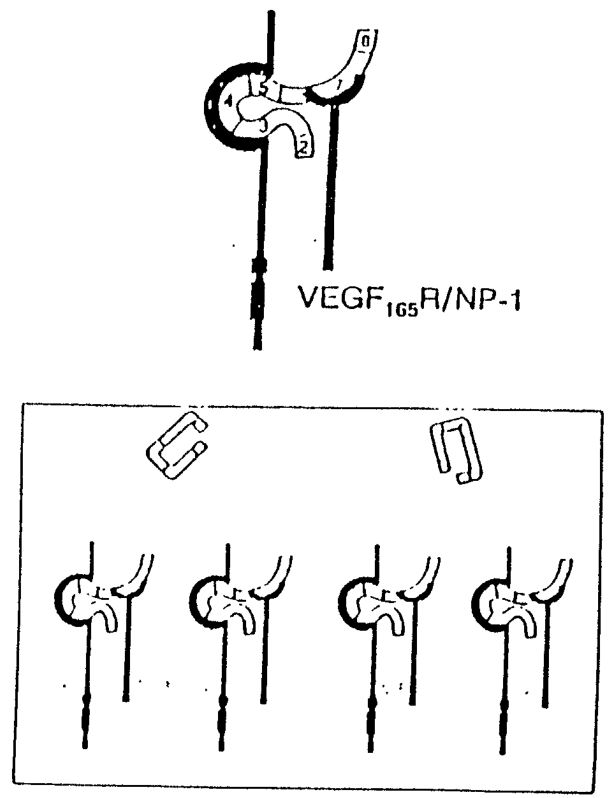

Figure 11C

KDR + $VEGF_{165}R/NP-1$ + Exon 7

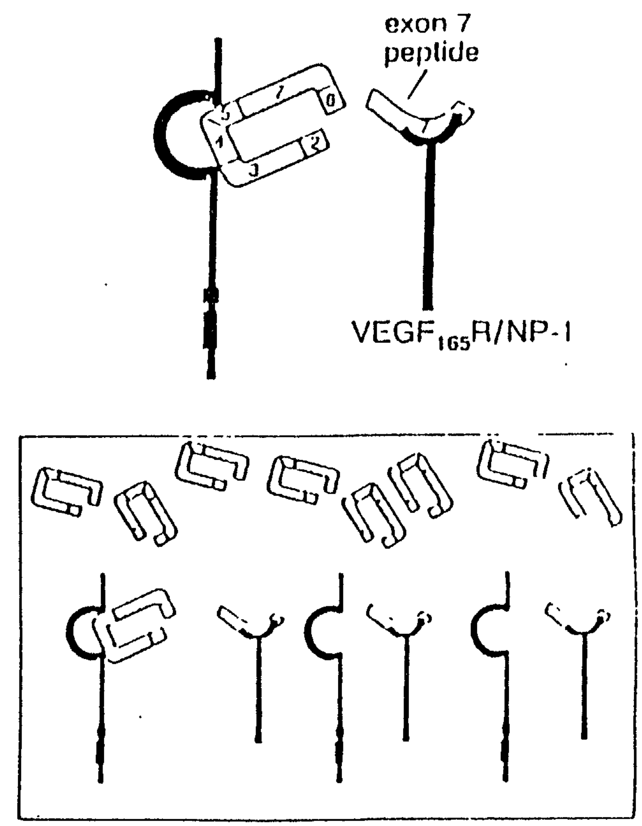

Figure 12

Human Neuropilin-2 amino acid sequence:

MDMFPLTWVFLALYFSRHQVRGQPDPPCGGRLNSKDAGYITSPGYPQDYPSHQN
CEWIVYAPEPNQKIVLNFNPHFEIEKHDCKYDFIEIRDGDSESADLLGKHCGNIAPP
TIISSGSMLYIKFTSDYARQGAGFSLRYEIFKTGSEDCSKNFTSPNGTIESPGFPEK
YPHNLDCTFTILAKPKMEIILQFLIFDLEHDPLQVGEGDCKYDWLDIWDGIPHVGPL
IGKYCGTKTPSELRSSTGILSLTFHTDMAVAKDGFSARYYLVHQEPLENFQCNVP
LGMESGRIANEQISASSTYSDGRWTPQQSRLHGDDNGWTPNLDSNKEYLQVDLR
FLTMLTAIATQGAISRETQNGYYVKSYKLEVSTNGEDWMVYRHGKNHKVFQANN
DATEVVLNKLHAPLLTRFVRIRPQTWHSGIALRLELFGCRVTDAPCSNMLGMLS
GLIADSQISASSTQEYLWSPSAARLVSSRSGWFPRIPQAQPGEEWLQVDLGTPK
TVKGVIIQGARGGDSITAVEARAFVRKFKVSYSLNGKDWEYIQDPRTQQPKLFEG
NMHYDTPDIRRFDPIPAQYVRVYPERWSPAGIGMRLEVLGCDWTDSKPTVETLG
PTVKSEETTTPYPTEEEATECGENCSFEDDKDLQLPSGFNCNFDFLEEPCGWMYD
HAKWLRTTWASSSSPNDRTFPDDRNFLRLQSDSQREGQYARLISPPVHLPRSPV
CMEFQYQATGGRGVALQVVREASQESKLLWVIREDQGGEWKHGRIILPSYDMEYQ
IVFEGVIGKGRSGEIAIDDIRISTDVPLENCMEPISAFAGENFKVDIPEIHEREGYED
EIDDEYEVDWSNSSSATSGSGAPSTDKEKSWLYTLDPILITIIAMSSLGVLLGAT
CAGLLLYCTCSYSGLSSRSCTTLENYNFELYDGLKHKVKMNHQKCCSEA*

Figure 13

```
gaattcggca cgaggggaaa ataaaagaga gaaaaacaca aagatttaaa caagaaacct      60
acgaacccag ctctggaaag agccaccttc tccaaaatgg atatgtttcc tctcacctgg     120
gttttcttag ccctctactt ttcaagacac caagtgagag gccaaccaga cccaccgtgc     180
ggaggtcgtt tgaattccaa agatgctggc tatatcacct ctcccggtta cccccaggac     240
tacccctccc accagaactg cgagtggatt gtttacgccc ccgaacccaa ccagaagatt     300
gtcctcaact tcaaccctca ctttgaaatc gagaagcacg actgcaagta tgactttatc     360
gagattcggg atggggacag tgaatccgca gacctcctgg gcaaacactg tgggaacatc     420
gccccgccca ccatcatctc ctcgggctcc atgctctaca tcaagttcac ctccgactac     480
gcccggcagg gggcaggctt ctctctgcgc tacgagatct tcaagacagg ctctgaagat     540
tgctcaaaaa acttcacaag ccccaacggg accatcgaat ctcctgggtt tcctgagaag     600
tatccacaca acttggactg cacctttacc atcctggcca aacccaagat ggagatcatc     660
ctgcagttcc tgatctttga cctggagcat gaccctttgc aggtgggaga gggggactgc     720
aagtacgatt ggctggacat ctgggatggc attccacatg ttggccccct gattggcaag     780
tactgtggga ccaaaacacc ctctgaactt cgttcatcga cggggatcct ctccctgacc     840
tttcacacgg acatggcggt ggccaaggat ggcttctctg cgcgttacta cctggtccac     900
caagagccac tagagaactt tcagtgcaat gttcctctgg gcatggagtc tggccggatt     960
gctaatgaac agatcagtgc ctcatctacc tactctgatg ggaggtggac ccctcaacaa    1020
agccggctcc atggtgatga caatggctgg acccccaact tggattccaa caaggagtat    1080
ctccaggtgg acctgcgctt tttaaccatg ctcacggcca tcgcaacaca gggagcgatt    1140
tccagggaaa cacagaatgg ctactacgtc aaatcctaca agctggaagt cagcactaat    1200
ggagaggact ggatggtgta ccggcatggc aaaaaccaca aggtatttca agccaacaac    1260
gatgcaactg aggtggttct gaacaagctc cacgctccac tgctgacaag gtttgttaga    1320
atccgccctc agacctggca ctcaggtatc gccctccggc tggagctctt cggctgccgg    1380
gtcacagatg ctccctgctc caacatgctg ggatgctct caggcctcat tgcagactcc     1440
cagatctccg cctcttccac ccaggaatac ctctggagcc ccagtgcagc ccgcctggtc    1500
agcagccgct cgggctggtt ccctcgaatc cctcaggccc agcccggtga ggagtggctt    1560
caggtagatc tgggaacacc caagacagtg aaaggtgtca tcatccaggg agcccgcgga    1620
ggagacagta tcactgctgt ggaagccaga gcatttgtgc gcaagttcaa agtctcctac    1680
agcctaaacg gcaaggactg ggaatacatt caggacccca ggacccagca gccaaagctg    1740
ttcgaaggga acatgcacta tgacacccct gacatccgaa ggtttgaccc cattccggca    1800
cagtatgtgc gggtataccc ggagaggtgg tcgccggcgg ggattgggat gcggctggag    1860
gtgctgggct gtgactggac agactccaag cccacggtag agacgctggg acccactgtg    1920
aagagcgaag agacaaccac cccctacccc accgaagagg aggccacaga gtgtggggag    1980
aactgcagct ttgaggatga caaagatttg cagctccctt cgggattcaa ttgcaacttc    2040
gatttcctcg aggagccctg tggttggatg tatgaccatg ccaagtggct ccggaccacc    2100
tgggccagca gctccagccc aaacgaccgg acgtttccag atgacaggaa tttcttgcgg    2160
ctgcagagtg acagccagag agagggccag tatgcccggc tcatcagccc ccctgtccac    2220
ctgccccgaa gcccggtgtg catggagttc cagtaccagg ccacgggcgg ccgcggggtg    2280
gcgctgcagg tggtgcggga agccagccag gagagcaagt tgctgtgggt catccgtgag    2340
gaccagggcg gcgagtggaa gcacgggcgg atcatcctgc ccagctacga catggagtac    2400
cagattgtgt tcgagggagt gatagggaaa ggacgttccg gagagattgc cattgatgac    2460
attcggataa gcactgatgt cccactggag aactgcatgg aacccatctc ggcttttgca    2520
ggtgagaatt ttaaagtgga catcccagaa atacatgaga gagaaggata tgaagatgaa    2580
attgatgatg aatacgaggt ggactggagc aattcttctt ctgcaacctc agggtctggc    2640
gcccccctcga ccgacaaaga aaagagctgg ctgtacaccc tggatcccat cctcatcacc   2700
atcatcgcca tgagctcact gggcgtcctc ctgggggcca cctgtgcagg cctcctgctc    2760
tactgcacct gttcctactc gggcctgagc tcccgaagct gcaccacact ggagaactac    2820
aacttcgagc tctacgatgg ccttaagcac aaggtcaaga tgaaccacca aaagtgctgc    2880
tccgaggcat gacggattgc acctgaatcc tatctgacgt ttcattccag caagaggggc    2940
tggggaagat tacatttttt tttcctttgg aaactgaatg ccataatctc gatcaaaccg    3000
atccagaata ccgaaggtat ggacaggaca gaaaagcgag tcgcaggagg aagggagatg    3060
cagccgcaca ggggatgatt accctcctag gaccgcggtg gctaagtcat tgcaggaacg    3120
gggctgtgtt ctctgctggg acaaaacagg agctcatctc tttggggtca cagttctatt    3180
ttgtttgtga gtttgtatta ttattattat tattattatt atattttatt tctttggtct    3240
gtgagcaact caaagaggca gaagaggaga atgactttc cagaatagaa gtggagcagt     3300
gatcattatt ctccgctttc tctttctaat caacacttga aagcaaagt gtcttttcag     3360
cctttccatc tttacaaata aaactcaaaa aagctgtcca gctt                    3404
```

Figure 14A

```
       247         256         265         274         283         292
5' ATG GAG AGG GGG CTG CCG CTC CTC TGC GCC GTG CTC GCC CTC GTC CTC GCC CCG
    M   E   R   G   L   P   L   L   C   A   V   L   A   L   V   L   A   P

       301         310         319         328         337         346
   GCC GGC GCT TTT CGC AAC GAT AAA TGT GGC GAT ACT ATA AAA ATT GAA AGC CCC
    A   G   A   F   R   N   D   K   C   G   D   T   I   K   I   E   S   P
                → a1

       355         364         373         382         391         400
   GGG TAC CTT ACA TCT CCT GGT TAT CCT CAT TCT TAT CAC CCA AGT GAA AAA TGC
    G   Y   L   T   S   P   G   Y   P   H   S   Y   H   P   S   E   K   C

       409         418         427         436         445         454
   GAA TGG CTG ATT CAG GCT CCG GAC CCA TAC CAG AGA ATT ATG ATC AAC TTC AAC
    E   W   L   I   Q   A   P   D   P   Y   Q   R   I   M   I   N   F   N

       463         472         481         490         499         508
   CCT CAC TTC GAT TTG GAG GAC AGA GAC TGC AAG TAT GAC TAC GTG GAA GTG TTC
    P   H   F   D   L   E   D   R   D   C   K   Y   D   Y   V   E   V   F

       517         526         535         544         553         562
   GAT GGA GAA AAT GAA AAT GGA CAT TTT AGG GGA AAG TTC TGT GGA AAG ATA GCC
    D   G   E   N   E   N   G   H   F   R   G   K   F   C   G   K   I   A

       571         580         589         598         607         616
   CCT CCT CCT GTT GTG TCT TCA GGG CCA TTT CTT TTT ATC AAA TTT GTC TCT GAC
    P   P   P   V   V   S   S   G   P   F   L   F   I   K   F   V   S   D

       625         634         643         652         661         670
   TAC GAA ACA CAT GGT GCA GGA TTT TCC ATA CGT TAT GAA ATT TTC AAG AGA GGT
    Y   E   T   H   G   A   G   F   S   I   R   Y   E   I   F   K   R   G

       679         688         697         706         715         724
   CCT GAA TGT TCC CAG AAC TAC ACA ACA CCT AGT GGA GTG ATA AAG TCC CCC GGA
    P   E   C   S   Q   N   Y   T   T   P   S   G   V   I   K   S   P   G
  a1 ←   → a2

       733         742         751         760         769         778
   TTC CCT GAA AAA TAT CCC AAC AGC CTT GAA TGC ACT TAT ATT GTC TTT GCG CCA
    F   P   E   K   Y   P   N   S   L   E   C   T   Y   I   V   F   A   P

       787         796         805         814         823         832
   AAG ATG TCA GAG ATT ATC CTG GAA TTT GAA AGC TTT GAC CTG GAG CCT GAC TCA
    K   M   S   E   I   I   L   E   F   E   S   F   D   L   E   P   D   S

       841         850         859         868         877         886
   AAT CCT CCA GGG GGG ATG TTC TGT CGC TAC GAC CGG CTA GAA ATC TGG GAT GGA
    N   P   P   G   G   M   F   C   R   Y   D   R   L   E   I   W   D   G

       895         904         913         922         931         940
   TTC CCT GAT GTT GGC CCT CAC ATT GGG CGT TAC TGT GGA CAG AAA ACA CCA GGT
    F   P   D   V   G   P   H   I   G   R   Y   C   G   Q   K   T   P   G

       949         958         967         976         985         994
   CGA ATC CGA TCC TCA TCG GGC ATT CTC TCC ATG GTT TTT TAC ACC GAC AGC GCG
    R   I   R   S   S   S   G   I   L   S   M   V   F   Y   T   D   S   A
```

Figure 14B

```
         1003          1012          1021          1030          1039          1048
ATA GCA AAA GAA GGT TTC TCA GCA AAC TAC AGT GTC TTG CAG AGC AGT GTC TCA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 I   A   K   E   G   F   S   A   N   Y   S   V   L   Q   S   S   V   S

         1057          1066          1075          1084          1093          1102
GAA GAT TTC AAA TGT ATG GAA GCT CTG GGC ATG GAA TCA GGA GAA ATT CAT TCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   D   F   K   C   M   E   A   L   G   M   E   S   G   E   I   H   S
   a2          b1

         1111          1120          1129          1138          1147          1156
GAC CAG ATC ACA GCT TCT TCC CAG TAT AGC ACC AAC TGG TCT GCA GAG CGC TCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   Q   I   T   A   S   S   Q   Y   S   T   N   W   S   A   E   R   S

         1165          1174          1183          1192          1201          1210
CGC CTG AAC TAC CCT GAG AAT GGG TGG ACT CCC GGA GAG GAT TCC TAC CGA GAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   L   N   Y   P   E   N   G   W   T   P   G   E   D   S   Y   R   E

         1219          1228          1237          1246          1255          1264
TGG ATA CAG GTA GAC TTG GGC CTT CTG CGC TTT GTC ACG GCT GTC GGG ACA CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 W   I   Q   V   D   L   G   L   L   R   F   V   T   A   V   G   T   Q

         1273          1282          1291          1300          1309          1318
GGC GCC ATT TCA AAA GAA ACC AAG AAG AAA TAT TAT GTC AAG ACT TAC AAG ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   A   I   S   K   E   T   K   K   K   Y   Y   V   K   T   Y   K   I

         1327          1336          1345          1354          1363          1372
GAC GTT AGC TCC AAC GGG GAA GAC TGG ATC ACC ATA AAA GAA GGA AAC AAA CCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   V   S   S   N   G   E   D   W   I   T   I   K   E   G   N   K   P

         1381          1390          1399          1408          1417          1426
GTT CTC TTT CAG GGA AAC ACC AAC CCC ACA GAT GTT GTG GTT GCA GTA TTC CCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 V   L   F   Q   G   N   T   N   P   T   D   V   V   V   A   V   F   P

         1435          1444          1453          1462          1471          1480
AAA CCA CTG ATA ACT CGA TTT GTC CGA ATC AAG CCT GCA ACT TGG GAA ACT GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   P   L   I   T   R   F   V   R   I   K   P   A   T   W   E   T   G

         1489          1498          1507          1516          1525          1534
ATA TCT ATG AGA TTT GAA GTA TAC GGT TGC AAG ATA ACA GAT TAT CCT TGC TCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 I   S   M   R   F   E   V   Y   G   C   K   I   T   D   Y   P   C   S
                                                           b1      b2

         1543          1552          1561          1570          1579          1588
GGA ATG TTG GGT ATG GTG TCT GGA CTT ATT TCT GAC TCC CAG ATC ACA TCA TCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   M   L   G   M   V   S   G   L   I   S   D   S   Q   I   T   S   S

         1597          1606          1615          1624          1633          1642
AAC CAA GGG GAC AGA AAC TGG ATG CCT GAA AAC ATC CGC CTG GTA ACC AGT CGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 N   Q   G   D   R   N   W   M   P   E   N   I   R   L   V   T   S   R

         1651          1660          1669          1678          1687          1696
TCT GGC TGG GCA CTT CCA CCC GCA CCT CAT TCC TAC ATC AAT GAG TGG CTC CAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   G   W   A   L   P   P   A   P   H   S   Y   I   N   E   W   L   Q

         1705          1714          1723          1732          1741          1750
ATA GAC CTG GGG GAG GAG AAG ATC GTG AGG GGC ATC ATC ATT CAG GGT GGG AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 I   D   L   G   E   E   K   I   V   R   G   I   I   I   Q   G   G   K

         1759          1768          1777          1786          1795          1804
CAC CGA GAG AAC AAG GTG TTC ATG AGG AAG TTC AAG ATC GGG TAC AGC AAC AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 H   R   E   N   K   V   F   M   R   K   F   K   I   G   Y   S   N   N
```

```
       1813        1822        1831        1840        1849        1858
GGC TCG GAC TGG AAG ATG ATC ATG GAT GAC AGC AAA CGC AAG GCG AAG TCT TTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   S   D   W   K   M   I   M   D   D   S   K   R   K   A   K   S   F

       1867        1876        1885        1894        1903        1912
GAG GGC AAC AAC AAC TAT GAT ACA CCT GAG CTG CGG ACT TTT CCA GCT CTC TCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 E   G   N   N   N   Y   D   T   P   E   L   R   T   F   P   A   L   S

       1921        1930        1939        1948        1957        1966
ACG CGA TTC ATC AGG ATC TAC CCC GAG AGA GCC ACT CAT GGC GGA CTG GGG CTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 T   R   F   I   R   I   Y   P   E   R   A   T   H   G   G   L   G   L

       1975        1984        1993        2002        2011        2020
AGA ATG GAG CTG CTG GGC TGT GAA GTG GAA GCC CCT ACA GCT GGA CCG ACC ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 R   M   E   L   L   G   C   E   V   E   A   P   T   A   G   P   T   T

       2029        2038        2047        2056        2065        2074
CCC AAC GGG AAC TTG GTG GAT GAA TGT GAT GAC GAC CAG GCC AAC TGC CAC AGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   N   G   N   L   V   D   E   C   D   D   D   Q   A   N   C   H   S

       2083        2092        2101        2110        2119        2128
GGA ACA GGT GAT GAC TTC CAG CTC ACA GGT GGC ACC ACT GTG CTG GCC ACA GAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   T   G   D   D   F   Q   L   T   G   G   T   T   V   L   A   T   E

       2137        2146        2155        2164        2173        2182
AAG CCC ACG GTC ATA GAC AGC ACC ATA CAA TCA GAG TTT CCA ACA TAT GGT TTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   P   T   V   I   D   S   T   I   Q   S   E   F   P   T   Y   G   F

       2191        2200        2209        2218        2227        2236
AAC TGT GAA TTT GGC TGG GGC TCT CAC AAG ACC TTC TGC CAC TGG GAA CAT GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 N   C   E   F   G   W   G   S   H   K   T   F   C   H   W   E   H   D

       2245        2254        2263        2272        2281        2290
AAT CAC GTG CAG CTC AAG TGG AGT GTG TTG ACC AGC AAG ACG GGA CCC ATT CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 N   H   V   Q   L   K   W   S   V   L   T   S   K   T   G   P   I   Q

       2299        2308        2317        2326        2335        2344
GAT CAC ACA GGA GAT GGC AAC TTC ATC TAT TCC CAA GCT GAC GAA AAT CAG AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 D   H   T   G   D   G   N   F   I   Y   S   Q   A   D   E   N   Q   K

       2353        2362        2371        2380        2389        2398
GGC AAA GTG GCT CGC CTG GTG AGC CCT GTG GTT TAT TCC CAG AAC TCT GCC CAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   K   V   A   R   L   V   S   P   V   V   Y   S   Q   N   S   A   H

       2407        2416        2425        2434        2443        2452
TGC ATG ACC TTC TGG TAT CAC ATG TCT GGG TCC CAC GTC GGC ACA CTC AGG GTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 C   M   T   F   W   Y   H   M   S   G   S   H   V   G   T   L   R   V

       2461        2470        2479        2488        2497        2506
AAA CTG CGC TAC CAG AAG CCA GAG GAG TAC GAT CAG CTG GTC TGG ATG GCC ATT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   L   R   Y   Q   K   P   E   E   Y   D   Q   L   V   W   M   A   I

       2515        2524        2533        2542        2551        2560
GGA CAC CAA GGT GAC CAC TGG AAG GAA GGG CGT GTC TTG CTC CAC AAG TCT CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 G   H   Q   G   D   H   W   K   E   G   R   V   L   L   H   K   S   L

       2569        2578        2587        2596        2605        2614
AAA CTT TAT CAG GTG ATT TTC GAG GGC GAA ATC GGA AAA GGA AAC CTT GGT GGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   L   Y   Q   V   I   F   E   G   E   I   G   K   G   N   L   G   G
```

Figure 14B

```
         2623                 2632                 2641                 2650                 2659                 2668
ATT GCT GTG GAT GAC ATT AGT ATT AAC AAC CAC ATT TCA CAA GAA GAT TGT GCA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 I   A   V   D   D   I   S   I   N   N   H   I   S   Q   E   D   C   A
                                                             C

         2677                 2686                 2695                 2704                 2713                 2722
AAA CCA GCA GAC CTG GAT AAA AAG AAC CCA GAA ATT AAA ATT GAT GAA ACA GGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 K   P   A   D   L   D   K   K   N   P   E   I   K   I   D   E   T   G

         2731                 2740                 2749                 2758                 2767                 2776
AGC ACG CCA GGA TAC GAA GGT GAA GGA GAA GGT GAC AAG AAC ATC TCC AGG AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   T   P   G   Y   E   G   E   G   E   G   D   K   N   I   S   R   K

         2785                 2794                 2803                 2812                 2821                 2830
CCA GGC AAT GTG TTG AAG ACC TTA GAT CCC ATC CTC ATC ACC ATC ATA GCC ATG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 P   G   N   V   L   K   T   L   D   P   I   L   I   T   I   I   A   M
                                          TM

         2839                 2848                 2857                 2866                 2875                 2884
AGT GCC CTG GGG GTC CTC CTG GGG GCT GTC TGT GGG GTC GTG CTG TAC TGT GCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 S   A   L   G   V   L   L   G   A   V   C   G   V   V   L   Y   C   A

         2893                 2902                 2911                 2920                 2929                 2938
TGT TGG CAT AAT GGG ATG TCA GAA AGA AAC TTG TCT GCC CTG GAG AAC TAT AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 C   W   H   N   G   M   S   E   R   N   L   S   A   L   E   N   Y   N
 TM        cyto

         2947                 2956                 2965                 2974                 2983                 2992
TTT GAA CTT GTG GAT GGT GTG AAG TTG AAA AAA GAC AAA CTG AAT ACA CAG AGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 F   E   L   V   D   G   V   K   L   K   K   D   K   L   N   T   Q   S

         3001                 3010
ACT TAT TCG GAG GCA TGA 3'   (SEQ ID NO:1)
--- --- --- --- --- ---
 T   Y   S   E   A   *       (SEQ ID NO:4)
           cyto                          2
```

Figure 15A

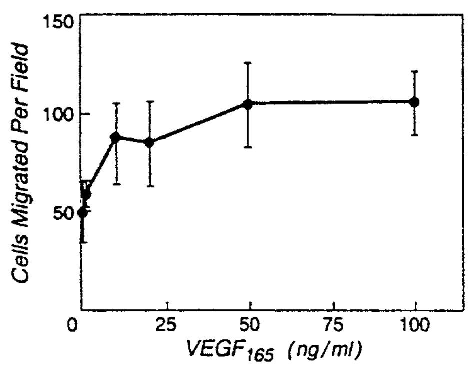

Figure 15B

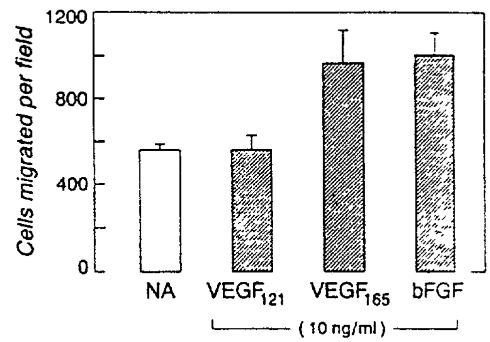

Figure 16A

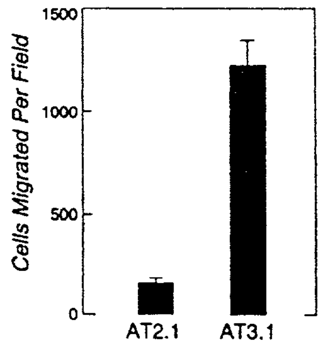

Figure 16B

Figure 16C

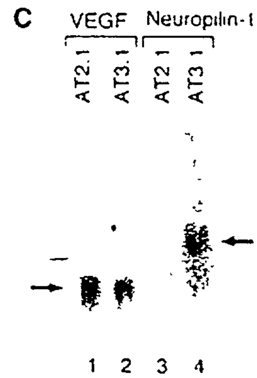

Figure 17A

Figure 17B

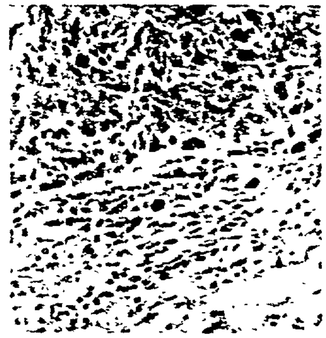

Figure 17C

Figure 18A

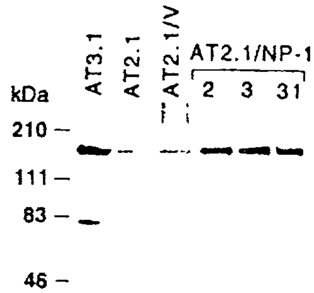

Figure 18B

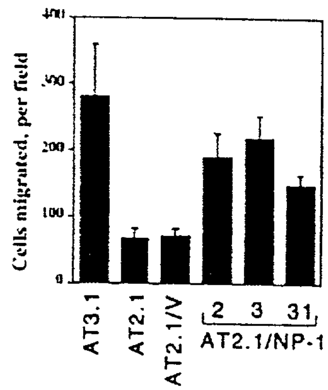

Figure 19

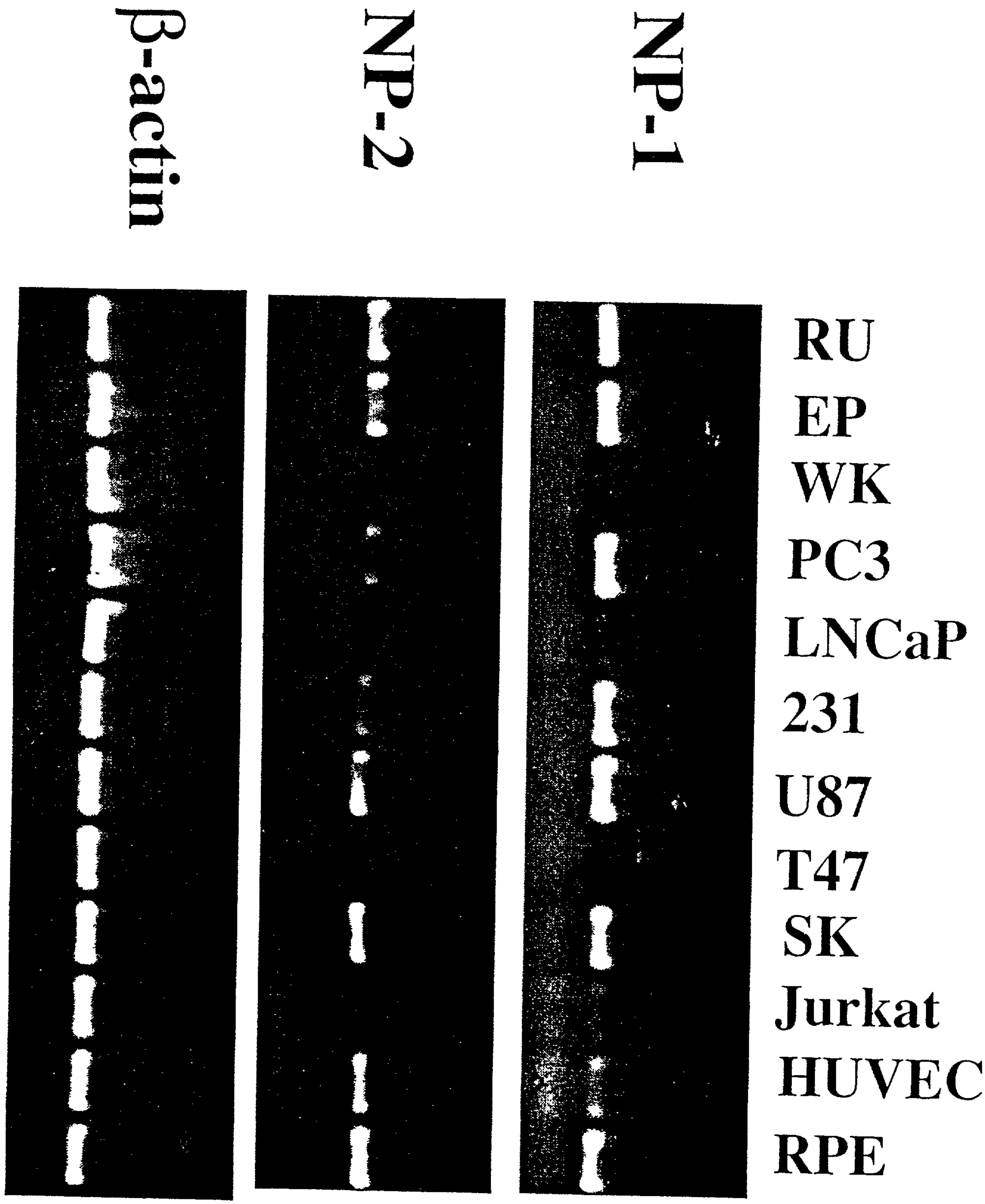
β-actin
NP-2
NP-1
RU
EP
WK
PC3
LNCaP
231
U87
T47
SK
Jurkat
HUVEC
RPE

Figure 20

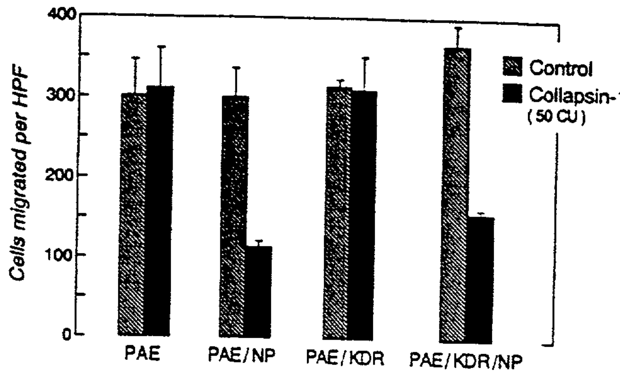

Figure 21A

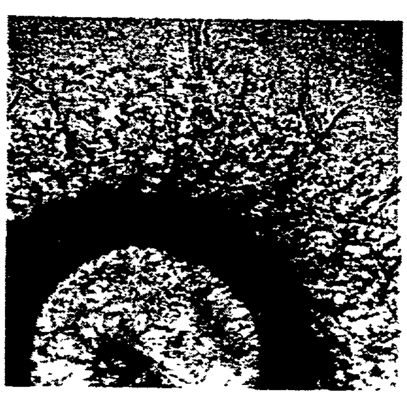

Figure 21B